UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE CIENCIAS QUÍMICAS
DEPARTAMENTO DE BIOQUÍMICA Y BIOLOGÍA MOLECULAR

TESIS DOCTORAL

INMUNOPROTEÓMICA DE LIGANDOS VIRALES DE HLA

MEMORIA PARA OPTAR AL GRADO DE DOCTORA
PRESENTADA POR

Elena Lorente Galán

Director

Daniel López Rodríguez

Madrid, 2015

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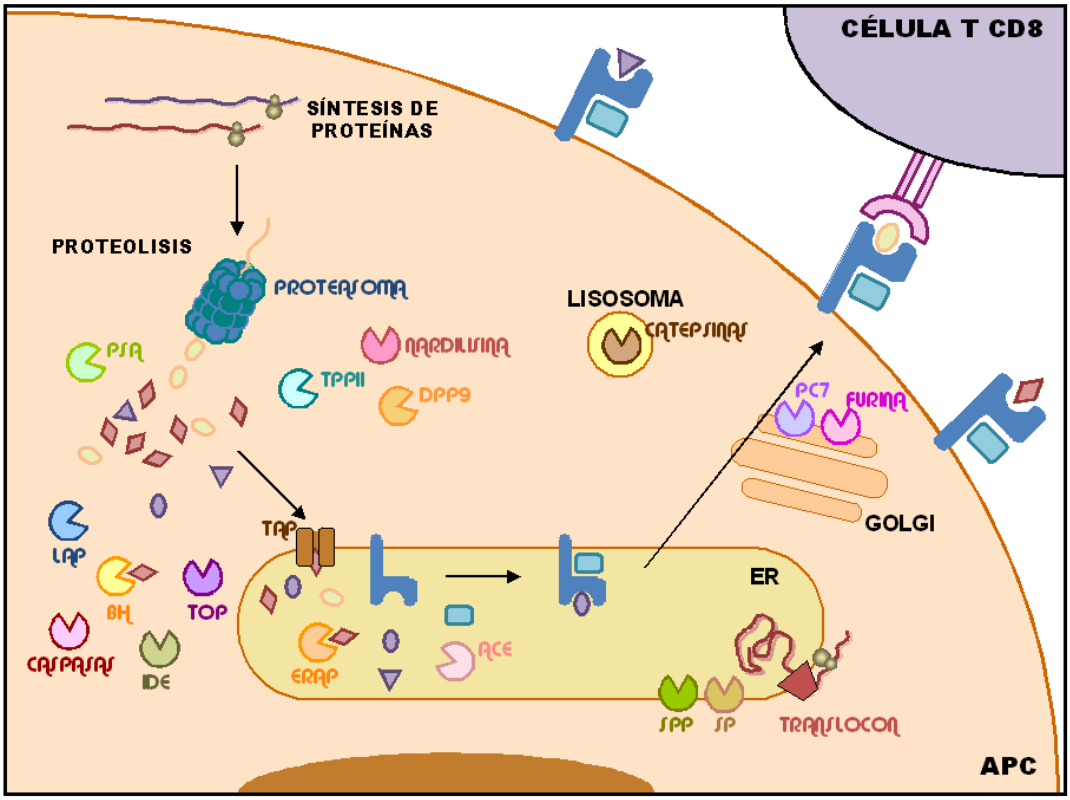

ELENA LORENTE GALÁN

TESIS DOCTORAL

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Esta Tesis Doctoral ha sido realizada en la Unidad de Inmunología Viral del Centro Nacional de Microbiología del Instituto de Salud Carlos III, bajo la dirección del Dr. Daniel López Rodríguez.
Tutor: Dr. Julián Gómez Gutiérrez

A mi familia

ÍNDICE

I.-ABREVIATURAS

Aa	Aminoácido
ACE	Enzima convertasa de angiotensina
ATP	Adenosin trifosfato
β_2m	β_2-microglobulina
BES	Bestatina
BFA	Brefeldina A
BH	Bleomicin hidrolasa
CMV	Citomegalovirus
CPV	Virus cowpox
CQ	Cloroquina
CTL	Linfocito T citotóxico CD8+
DNA	Ácido desoxirribonucleico
DPP9	Dipeptidilpeptidasa 9
EBV	Virus de Epstein-Barr
ER	Retículo endoplasmático
ERAP	Aminopeptidasa del RE
HIV	Virus de la inmunodeficiencia humana
HLA	Antígeno leucocitario humano
HSV	Virus del herpes simple
IDE	Enzima degradadora de insulina
IFN	Interferón
kDa	Kilodalton
LAP	Leucín aminopeptidasa
MHC	Complejo principal de histocompatibilidad
PC7	Proprotein convertasa 7
PDI	Proteína disulfuro isomerasa
PSA	Aminopeptidasa sensible a puromicina
SP	Peptidasa señal
SPP	Peptidasa del péptido señal
TAP	Transportador asociado al procesamiento de antígeno
TAPL	Transportador lisosomal
TCR	Receptor de células T
TOP	Oligopeptidasa thimet
TPPII	Tripeptidil peptidasa II

II.-SUMMARY

Title: Immunoproteomics of viral HLA-ligands

Introduction

CD8 T lymphocytes screen peptides displayed at the plasma membrane by HLA class I molecules (HLA-I). Most of these peptides are produced from cytosolic proteolysis by the proteasome and other proteases, such as tripeptidyl peptidase II (1), puromycin-sensitive aminopeptidase (2), insulin-degrading enzyme (3), nardilysin, thimet oligopeptidase (4), and caspases (5). These peptides are transported by TAP into the endoplasmic reticulum (ER). There, in many cases, subsequent N-terminal trimming by the metalloaminoprotease ERAP1 is required for peptide binding to nascent HLA-I molecules (6). The stable peptide-HLA complexes are exported to the cell membrane where they are exposed for cytotoxic $CD8^+$ T lymphocyte recognition (7).

Non-functional TAP complexes, which can be produced by mutations in the TAP gene, have been described in both mice (8) and humans (9). Patients with this HLA-I deficiency have a reduced functional $CD8^+$ population but may appear asymptomatic for long periods of time with only a limited susceptibility to chronic respiratory bacterial infections. Thus, their immune systems must be reasonably efficient and, in addition to different unaltered layered defenses, it remains possible that the reduced cytolytic $CD8^+$ $\alpha\beta$ T subpopulation that is specific for TAP-independent antigens may contribute to immune defenses that protect against severe infections in these individuals. The implication of TAP-independent pathways in the generation of particular but diverse pathogenic epitopes was previously reported (10-12). However, there has been a marked absence of methodical studies of TAP-independent epitopes and ligands restricted by different HLA molecules in cells infected with a single virus. The identified proteases involved in the generation of specific ligands in TAP-deficient cells include ER signal peptidase (SP) (13,14), ER signal peptide peptidase (SPP) (15), trans-Golgi network furin (16), and lysosomal cathepsins (17). Nevertheless, systematic studies of TAP-independent pathways involved in the generation of the overall peptide repertoire associated with different HLA-I molecules have not been reported.

The eradication of smallpox, a disease caused by variola major virus, started with the early empirical, cross-protective vaccination with cowpox virus (18). The role of cellular responses in this cross-protection is well documented (19,20). A cowpox protein CPXV12, present in several strains, inhibits peptide translocation by TAP, thereby interfering with MHC class I-peptide complex formation (21). Thus, the possible contribution of $CPXV12^+$ cowpox virus strains to early vaccination must not be discarded above all when some of

these cowpox virus strains were isolated in the southern region of England, roughly where the early vaccination works occurred.

Hypothesis

Patients with mutations in the TAP gene may appear asymptomatic for long periods of time with only a limited susceptibility to chronic respiratory bacterial infections, thus their immune systems must be reasonably efficient and, in addition to different unaltered layered defenses, it remains possible that the reduced cytolytic $CD8^+$ $\alpha\beta$ T subpopulation that is specific for TAP-independent antigens may contribute to immune defenses that protect against severe infections in these individuals.

The identification of TAP-independent epitopes conserved among orthopoxviruses could be relevant to the study of the mechanisms of early empirical vaccination against smallpox disease performed with $CPXV12^+$ cowpox virus strains.

Objetives

The identification of viral and endogenous ligands presented by several common HLA-I molecules in infected TAP-deficient cells and the characterization of their antigen processing pathways.

Materials and methods

The presentation of viral and endogenous HLA-I ligands was examined by high-throughput mass spectrometry analysis of the peptides isolated from the peptide-HLA pools of healthy or vaccinia-infected cells. HLA-bound peptides were isolated from 4 E10 healthy or vaccinia-infected TAP-deficient cells as previously described (22).

The $CD8^+$ immune response, after infection with vaccinia virus, was evaluated in HLA-A*02 or -B*07 transgenic mice. Vaccinia-specific $CD8^+$ T lymphocytes for the different identified TAP-independent ligands were generated in vitro and the characterization of the antigen processing pathways of these ligands in TAP-competent cells was performed.

Results

This study identified 334 ligands from 182 human proteins, 13 ligands from ten different vaccinia proteins and 1 from the gp160 of HIV presented by six different HLA-A, -B, -C and -E class I molecules generated by TAP-independent pathways. Three of these vaccinia HLA-ligands were presented by two different HLA-I alleles, and, as a result, 16 different HLA-peptide complexes were detected.

The repertoire of endogenous TAP-independent peptides identified by mass spectrometry revealed increased peptide lengths and a lack of strict binding motifs in all

HLA-I molecules studied. The TAP-independent peptidome arose from 182 parental proteins, the majority of which yielded one HLA ligand. In contrast, TAP-independent antigen processing of very few cellular proteins generated multiple HLA ligands. Comparison between TAP-independent peptidome and proteome of several subcellular locations suggests that the secretory vesicle-like organelles could be a relevant source of parental proteins for TAP-independent HLA ligands. Finally, a predominant endoproteolytic peptidase specificity for Arg/Lys or Leu/Phe residues in the P1 position of the scissile bond was found for the immunoprecipitated TAP-independent ligands.

In addition, the generation of the overall peptide repertoire associated with four different HLA-I molecules (HLA-A*02, -B*27, -B*51 and -C*01) in the same TAP-deficient cells was studied. Using different protease inhibitors, four different proteolytic specificities were identified. The inhibition obtained in all HLA-class I alleles examined in presence of BFA indicates that most of the TAP-independent HLA-bound peptides were endogenously processed. The surface levels of HLA-A*02 and -B*51 class I were dependent of proteasome, metallo-aminopeptidases (probably ERAP), and SPP activities. Metallo-aminopeptidases and lysosomal antigen processing are relevant for generating HLA-B*27 ligands. By contrast, none of the compounds used in this study decreased HLA-C*01 surface expression.

In the repertoire of TAP-independent peptides analyzed by mass spectrometry eleven vaccinia viral ligands were identified. In addition to the high-affinity ligands, one low-affinity peptide restricted by each of the HLA-A, -B, and -C class I molecules was identified. Both high- and low-affinity ligands generated long-term memory CTL responses to vaccinia virus in an HLA-A*02^{+} transgenic mouse model. The processing and presentation of two vaccinia virus HLA-A*02-restricted antigens took place via proteasomal and nonproteasomal pathways. This nonproteasomal pathways were blocked in infected TAP-suficient cells with chemical inhibitors specific for different subsets of metalloproteinases.

In addition, two vaccinia-derived TAP-independent HLA-B*07-restricted epitopes were identified using a TAP-independent polyclonal vaccinia virus-polyspecific CD8^{+} T cell line. The presentation of these epitopes in TAP-suficient cells occurs via complex antigen-processing pathways involving the proteasome and/or different subsets of metalloproteinases (amino-, carboxy-, and endoproteases), which were blocked in infected cells with specific chemical inhibitors.

The comparison of the sequences of the vaccinia virus ligands identified by mass spectrometry revealed a high degree of conservation of the ligands among orthopoxviruses. Twelve of these 13 ligands are almost fully conserved in the variola major and minor

viruses. Only 60% of the previously described TAP-dependent vaccinia virus epitopes are conserved in the variola proteome (23-25), indicating that TAP-independent vaccinia ligands are more conserved between immunogenic and pathological poxviruses than TAP-dependent epitopes.

Discussion

The global picture emerging from the analysis of the endogenous TAP-independent pathways is consistent with the following model. Mostly HLA-A*02 and -B*51-restricted ligands are processed by the Spase and proteasome in accordance with previous studies (13,14,26), and the B*27-restricted peptidome by lysosomal peptidases. Besides, endoproteolytic peptidases, exhibiting specificity for Arg/Lys or Phe/Leu in the P1 position of the scissile bond, play an important role in the generation of many ligands associated with the four HLA-I alleles studied herein. However, some TAP-independent ligands must be produced by other yet uncharacterized protease activities. In addition, some of these HLA-I-restricted peptides need further trimming by aminopeptidases and carboxypeptidases. In summary, different and complex processing pathways involving at least six diverse proteolytic specificities in miscellaneous subcellular locations are required to generate the HLA-I peptide repertoire in TAP-deficient cells.

In addition, an exceptional diversity of TAP-independent ligands from vaccinia-infected cells has been identified in all HLA-I alleles studied. This broad complexity of viral peptide-MHC complexes could help to explain why TAP-deficient patients do not seem particularly susceptible to viral infections and may appear asymptomatic for long periods of their lives.

Only the HLA-I epitopes conserved between the vaccine virus (cowpox or vaccinia) and the pathogenic variola virus were responsible for the crossreactive protection in individuals exposed to variola virus. In this study the TAP-independent ligands were found to be highly conserved among orthopoxviruses, which could entail the success of early empirical vaccination against smallpox disease with CPXV12^{+} cowpox virus strains.

Conclusions

The data demonstrate that different and complex allele-dependent processing pathways are involved in the generation of the HLA-I peptide repertoire in TAP-deficient cells.

These findings show the possible contribution of TAP-independent antigen processing pathways to CD8 T-cell response and crossprotection from infection with variola.

The simultaneous presentation of the elements of this broad complexity of viral peptide-MHC complexes can help to explain why TAP-deficient patients do not seem particularly susceptible to viral infections and may appear asymptomatic for long periods of their lives.

III.-RESUMEN

Título: Inmunoproteómica de ligandos virales de HLA

Introducción

Los linfocitos T $CD8^+$ reconocen péptidos patogénicos presentados por las moléculas de HLA de clase I (HLA-I) en la superficie celular. La mayoría de estos péptidos son generados en el citosol por el proteasoma y por otras proteasas, como la tripeptidil peptidasa II (1), la aminopeptidasa sensible a puromicina (2), la enzima degradadora de insulina (3), la nardilisina (4), la oligopeptidasa thimet (4) y las caspasas (5) y posteriormente son transportados al retículo endoplásmico (ER) por TAP. Allí, en muchos casos, se requiere un recorte de su extremo amino terminal por la metaloaminoproteasa ERAP1 previo a su unión con la molécula de HLA-I recién sintetizada (6). Los complejos de HLA-péptido estables son transportados a la membrana celular, donde quedan expuestos al reconocimiento de los linfocitos T $CD8^+$ citotóxicos (7).

Se han descrito, tanto en ratón (8) como en humanos (9), complejos de TAP no funcionales que pueden generarse por mutaciones en el gen de TAP. Los pacientes con esta deficiencia de HLA-I tienen una población de $CD8^+$ reducida pero funcional y pueden permanecer asintomáticos durante largos periodos de tiempo, con solo una cierta susceptibilidad a infecciones bacterianas crónicas de las vías respiratorias. Por tanto, su sistema inmune debe ser lo suficientemente eficiente y es posible que la reducida subpoblación citolítica T $CD8^+$ $\alpha\beta$, específica para los antígenos independientes de TAP, pueda contribuir a las defensas inmunitarias que protegen contra infecciones graves en estos individuos junto con las restantes poblaciones inmunes inalteradas. Las vías independientes de TAP contribuyen a la generación de diversos epítopos particulares de patógenos (10-12). Sin embargo, no se han realizado estudios exhaustivos para la identificación de epítopos o ligandos independientes de TAP restringidos por diferentes moléculas HLA-I en células infectadas con un mismo virus. Se han identificado varias proteasas que participan en la generación de ligandos específicos en las células deficientes en TAP como la peptidasa señal (SP) (13,14) y la peptidasa del péptido señal (SPP) (15) en el ER, la furina en el trans-Golgi network (16) y las catepsinas en lisosomas (17). No obstante, tampoco se han realizado estudios sistemáticos de las vías independientes de TAP involucradas en la generación del repertorio peptídico global asociado a diversas moléculas HLA-I.

La erradicación de la viruela comenzó inicialmente con una vacunación empírica con el virus cowpox, inmunización que fue capaz de generar una protección cruzada frente al patógeno (18). El papel de la respuesta inmune celular en esta protección cruzada está bien documentado (19,20). La proteína CPXV12, presente en varias cepas de cowpox,

inhibe la translocación de péptidos por TAP, interfiriendo con la formación de complejos MHC de clase I-péptido (21). Por lo tanto, como algunas de estas cepas se han aislado en la región del sur de Inglaterra, en donde se produjeron las primeras vacunaciones, es posible que las cepas CPXV12$^+$ del virus de cowpox contribuyeran al éxito inicial de la vacunación.

Hipótesis

Los pacientes con mutaciones en el gen TAP pueden parecer asintomáticos durante largos períodos de tiempo con sólo una susceptibilidad limitada a las infecciones bacterianas crónicas del sistema respiratorio, por lo tanto su sistema inmunológico debe ser razonablemente eficiente y es posible que la reducida subpoblación citolítica T CD8$^+$ $\alpha\beta$, específica para los antígenos independientes de TAP, pueda contribuir a la protección contra infecciones graves en estos individuos junto con las restantes poblaciones inmunes que no se encuentran alteradas.

La identificación de epítopos independientes de TAP conservados entre los orthopoxvirus podría ser relevante para el estudio de los mecanismos de la vacunación inicial contra la viruela realizada con cepas del virus cowpox que codifican para la proteína CPXV12$^+$.

Objetivos

La identificación de ligandos virales y endógenos, presentados por varios antígenos HLA comunes en la población, en células deficientes en TAP infectadas con vaccinia y la caracterización de sus vías de procesamiento antigénico.

Materiales y métodos

Se estudió la presentación de ligandos de HLA-I virales y endógenos mediante el análisis por espectrometría de masas de alta resolución de los péptidos aislados de los complejos péptido-HLA tanto de células infectadas como no infectadas por el virus vaccinia. Los péptidos unidos a HLA se aislaron a partir de 4 E10 de células deficientes en TAP infectadas o no infectadas con vaccinia como se describió previamente (22).

También se evaluó la respuesta inmune mediada por los linfocitos T CD8$^+$, generada tras la infección con el virus de vaccinia, en ratones transgénicos para HLA-A*02 o -B*07. Además, se generaron líneas de linfocitos T CD8$^+$ específicas frente a los diferentes ligandos independientes de TAP identificados con objeto de caracterizar las vías de procesamiento de antígeno implicadas en la generación de estos ligandos en células competentes en TAP.

Resultados

En este estudio se han identificado 334 ligandos derivados de 182 proteínas humanas, 13 ligandos de diez proteínas de vaccinia diferentes y 1 de la gp160 del VIH, presentados por seis moléculas de clase I HLA-A, -B, -C y -E distintas que son generados por vías deficientes en TAP. Tres de estos ligandos de HLA-I de vaccinia fueron presentados por dos alelos de HLA-I diferentes, por lo que se detectaron en total 16 complejos péptido-HLA diferentes.

El repertorio peptídico endógeno presentado independientemente de TAP, identificado mediante espectrometría de masas, reveló un incremento en la longitud y una falta de motivos de anclaje estrictos en los péptidos presentados por todas las moléculas de HLA-I estudiadas. El peptidoma independiente de TAP derivó de 182 proteínas parentales, la mayoría de las cuales contribuyó con un único ligando de HLA-I. Por el contrario, el procesamiento antigénico independiente TAP de unas pocas proteínas celulares generó múltiples ligandos de HLA-I. La comparación entre el peptidoma independiente de TAP y el proteoma presente en varias localizaciones subcelulares sugiere que orgánulos similares a las vesículas secretorias podrían ser una fuente importante de las proteínas parentales de los ligandos de HLA-I independientes de TAP. Por último, se identificó una actividad peptídica predominante, específica para residuos Arg / Lys o Leu / Phe en la posición P1 del sitio de corte de la enzima implicada en la generación de los ligandos independientes de TAP caracterizados.

Además, se estudió la generación del repertorio péptido asociado a cuatro moléculas de HLA-I diferentes (HLA-A*02, -B*27, -B*51 y -C*01) presentadas en las misma línea celular deficiente en TAP. El empleo de inhibidores de la diversas proteasas permitió la identificación de cuatro especificidades proteolíticas diferentes. La inhibición de la expresión de todos los alelos de HLA-I en presencia de brefeldina (BFA) indica que la mayoría de los ligandos de HLA independientes de TAP fueron procesados endógenamente. Los niveles de expresión de HLA-A*02 y -B*51 dependían de las actividades del proteasoma, de metalo-aminopeptidasas (probablemente ERAP) y de la SPP. Mientras que las metalo-aminopeptidasas y el procesamiento antigénico lisosomal son relevantes para la generación de muchos de los ligandos de HLA-B*27. Por el contrario, ninguno de los compuestos utilizados en este estudio disminuyó la expresión de HLA-C*01 en superficie.

En el repertorio peptídico analizado por espectrometría de masas también se identificaron 11 ligandos virales de vaccinia presentados independientemente de TAP. Además de diversos ligandos de alta afinidad, se identificó un péptido de baja afinidad

restringido por cada una de las moléculas de clase I, HLA-A, -B y -C. Tanto los ligandos de alta como los de baja afinidad generaron respuestas de CTLs de memoria a largo plazo frente al virus vacunal en el modelo de ratón transgénico para HLA-A*02. El procesamiento y la presentación de dos de los ligandos de vaccinia restringidos por HLA-A*02 se llevaron a cabo mediante dos vías de procesamiento, una dependiente y otra independiente de la actividad del proteasoma. La vía de procesamiento independiente del proteasoma se bloqueó con inhibidores químicos específicos para distintos subtipos de metaloproteasas en las células suficientes en TAP infectadas.

Además, se identificaron otros dos epítopos independientes de TAP, restringidos por HLA-B*07 y derivados de vaccinia, mediante el empleo de una línea celular T $CD8^+$ policlonal poliespecífica generada frente a epítopos independientes de TAP de este virus. La presentación de estos epítopos en las células suficientes en TAP se produce a través de vías de procesamiento de antígeno complejas que implican al proteasoma y/o diferentes subtipos de metaloproteasas (amino, carboxi y endoproteasas), ya que fueron bloqueadas en las células infectadas en presencia de los inhibidores químicos específicos.

La comparación de las secuencias de los ligandos identificados por espectrometría de masas del virus vaccinia reveló un elevado grado de conservación entre los orthopoxvirus. Doce de estos 13 ligandos están casi totalmente conservados en el virus de la viruela. Mientras que solo el 60% de los epítopos dependientes de TAP del virus vaccinia descritos anteriormente se conservan en el proteoma de viruela (23-25), lo que indica que los ligandos de vaccinia independientes de TAP están más conservados entre los poxvirus inmunogénicos y patogénicos que los epítopos dependientes de TAP.

Discusión

La imagen global emergente del análisis de las vías independientes de TAP implicadas en la generación del repertorio endógeno es consistente con el siguiente modelo. La mayoría de los ligandos restringidos por HLA-A*02 y -B*51 son procesados por la SP y por el proteasoma, de acuerdo con estudios previos (13,14,26) y el peptidoma restringido por HLA-B*27 por peptidasas lisosomales. Además, peptidasas endoproteolíticas, con especificidad por Arg / Lys o Phe / Leu en la posición P1 del enlace escindible, juegan un papel importante en la generación de muchos ligandos asociados con los cuatro alelos HLA-I estudiados en el presente trabajo. Sin embargo, algunos ligandos independientes de TAP deben ser producidos por otras actividades proteolíticas aún no caracterizadas. Además, algunos de estos péptidos presentados por HLA-I requieren de un recorte adicional por aminopeptidasas y carboxipeptidasas. En resumen, se requieren diferentes y complejas vías de procesamiento que implican, al menos, seis especificidades proteolíticas

diferentes en localizaciones subcelulares diversas, para generar el repertorio peptídico de HLA-I en las células deficientes en TAP.

Además, se ha identificado una diversidad excepcional de ligandos independientes de TAP en todos los alelos de HLA clase I estudiados en células infectadas con vaccinia. Esta gran variedad de complejos MHC-péptido viral podría ayudar a explicar por qué los pacientes deficientes en TAP no parecen particularmente susceptibles a las infecciones virales y se muestran asintomáticos durante largos períodos de sus vidas.

Solo los epítopos de HLA-I conservados entre el virus vacunal (cowpox o vaccinia) y el virus patogénico pudieron ser responsables de la protección por reactividad cruzada en personas expuestas al virus de la viruela. En este estudio se ha encontrado que los ligandos independientes de TAP se encuentran altamente conservados entre los ortopoxvirus, lo que podría explicar el éxito inicial de la vacunación contra la viruela con las cepas de cowpox CPXV12^{+}.

Conclusiones

Los datos demuestran la existencia de vías de procesamiento diferentes y complejas involucradas en la generación del repertorio peptídico de HLA-I en las células deficientes en TAP, que son dependientes del alelo analizado.

Estos hallazgos muestran que las vías de procesamiento antigénico independientes de TAP podrían contribuir a la respuesta de las células T CD8^{+} y a la protección cruzada frente a viruela.

La presentación simultánea de diversos complejos de MHC-péptido viral puede ayudar a explicar por qué los pacientes deficientes en TAP no parecen particularmente susceptibles a las infecciones virales y pueden mostrarse asintomáticos durante largos períodos de sus vidas.

IV.-INTRODUCCIÓN

1.- El sistema inmune y los patógenos

La respuesta inmune adaptativa mediada por los linfocitos B (humoral) y T (celular) reconoce proteínas derivadas de agentes infecciosos, como las bacterias y los virus. La respuesta humoral es efectiva contra antígenos extracelulares, ya que los anticuerpos liberados por los linfocitos B son capaces de unirse a la conformación nativa de las proteínas del microorganismo invasor. En cambio, los linfocitos T reconocen péptidos derivados de las proteínas del patógeno, unidos al complejo principal de histocompatibilidad (MHC, del inglés “major histocompatibility complex”), en la superficie de la célula presentadora de antígeno.

2.- Vía clásica de procesamiento antigénico

2.1.- Procesamiento citosólico

Las moléculas de MHC de clase I (MHC-I) presentan péptidos a los linfocitos T citotóxicos $CD8^+$ (CTLs) en la superficie de la mayoría de las células nucleadas del organismo. Estos péptidos son generados mayoritariamente en el citosol por el proteasoma a partir de proteínas celulares o derivadas del patógeno (27). El proteasoma degrada sus sustratos a péptidos de entre 3-20 aminoácidos (Aas) (28), siendo la mayoría de ellos procesados posteriormente por aminopeptidasas hasta generar aminoácidos libres que son reciclados en la síntesis de nuevas proteínas. Se han identificado diversas endo- y exoproteasas citosólicas capaces de sustituir o complementar la actividad del proteasoma (Tabla 1).

2.1.1.- Proteasoma

El proteasoma es un complejo proteolítico multicatalítico responsable de la degradación de la mayor parte de las proteínas intracelulares en los mamíferos, localizado tanto en el núcleo como en el citoplasma celular (28,29). El proteasoma está compuesto por una subunidad proteolítica central, conocida como proteasoma 20S y dos reguladoras o 19S que se unen a cada extremo del núcleo catalítico (30) (Figura 1A).

Cada complejo 19S está compuesto por casi 20 subunidades diferentes que forman dos subestructuras, la tapa y la base (31). La tapa está constituida por 8 subunidades no ATPasas, situadas hacia el exterior, que son necesarias para la eliminación de la ubiquitina de las proteínas poliubiquitinadas. La ubiquitina es una proteína de pequeño peso molecular que sirve de señal al proteasoma para identificar las proteínas que han de ser degradadas. Las principales funciones de la base son desnaturalizar los sustratos y permitir su entrada al 20S. Todos estos procesos requieren un aporte metabólico de energía, por lo que 6 de las 9 subunidades que constituyen la base del 19S son ATPasas (32).

El proteasoma 20S se compone de cuatro anillos apilados formando una estructura cilíndrica. Cada uno de los dos anillos externos está formado por 7 subunidades α diferentes, que permiten la entrada de los sustratos e interaccionan con complejos reguladores, y los dos anillos centrales por otras 7 subunidades β diferentes encargadas de llevar a cabo la degradación de los sustratos. En mamíferos, las subunidades catalíticas son β1, β2 y β5 y presentan actividades proteolíticas distintas: caspasa, corte tras residuos ácidos, tríptica, tras residuos básicos, y quimiotríptica, tras residuos hidrofóbicos, respectivamente (33). La estimulación con diversas citoquinas, especialmente interferón-γ (IFN-γ), induce la expresión de tres subunidades catalíticas alternativas (LMP2 (β_{1i}), MECL1 (β_{2i}) y LMP7 (β_{5i})) que actúan tras residuos hidrofóbicos, básicos y neutros e hidrofóbicos voluminosos respectivamente, y sustituyen a sus homólogas constitutivas para formar el denominado inmunoproteasoma (34,35). Estas nuevas subunidades dan al inmunoproteasoma una especificidad de corte parcialmente distinta a la del proteasoma 20S (36,37), alterando el conjunto del repertorio peptídico generado (38). En los últimos años se han identificado proteasomas intermedios que presentan una mezcla de las subunidades del proteasoma constitutivo y del inmunoproteasoma (39,40).

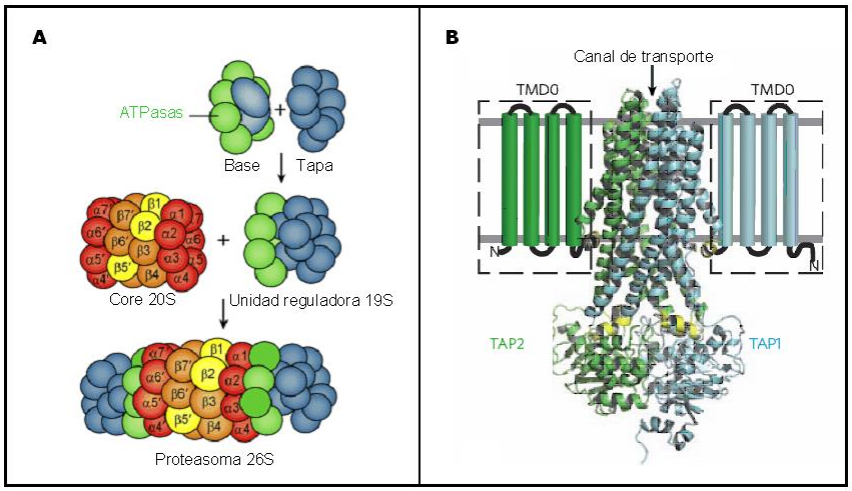

Figura 1.- Estructura del proteasoma 26S y de TAP

El núcleo catalítico o proteasoma 20S se representa en rojo (subunidades α), naranja (subunidades β no catalíticas) y amarillo (subunidades β catalíticas) y el complejo regulador 19S en azul (tapa) y verde (base) (panel A). En el panel B se muestra la estructura del complejo TAP, con las dos subunidades homólogas, TAP1 (azul) y TAP2 (verde). Imagen adaptada a partir de (41)

2.1.2.- Aminopeptidasas citosólicas

- La tripeptidil peptidasa II (TPPII): es una serín-aminopeptidasa que recorta tripéptidos del extremo amino de péptidos largos. Por ello, se postula que podría ser la encargada de procesar los productos de mayor tamaño generados por el proteasoma (42,43). Además, la TPPII tiene actividad endoproteolítica de tipo tripsina, pudiendo sustituir en ocasiones parcialmente la actividad endoproteolítica del proteasoma (44). Es esencial para la generación de varios epítopos de CTLs, aunque también puede ser destructiva para otros (1,45-50). En los ratones transgénicos deficientes en TPPII se ha observado un moderado aumento de la expresión en superficie de MHC-I, lo que sugiere una actividad global de esta enzima ligeramente destructiva (51,52).

- Dipeptidilpeptidasa 9 (DPP9): es una serínproteasa que recorta tras el residuo prolina. Se ha descrito que esta enzima degrada un epítopo tumoral generado por la TPPII (53).

- La bleomicin hidrolasa (BH) y la leucín-aminopeptidasa (LAP): la BH es una cistein-proteasa, mientras que LAP es una metaloproteasa con preferencia por el recorte de péptidos con Aas hidrofóbicos en el extremo amino (54). La respuesta inmune mediada por los linfocitos T $CD8^+$ en los ratones $BH^{-/-}$ o $LAP^{-/-}$ es normal para todos los epítopos analizados y no parece que ninguna de las dos sea esencial para la generación o destrucción de los ligandos de MHC-I (55,56). Sin embargo, se ha observado que la respuesta frente a un epítopo de la glicoproteína del virus de la coriomeningitis linfocítica aumenta significativamente en ratones $LAP^{-/-}BH^{-/-}$, por lo que ambas enzimas podrían tener un papel destructor de ligandos de MHC-I (55).

- Aminopeptidasa sensible a puromicina (PSA): es una metalopeptidasa cuya expresión no se ve alterada por el tratamiento con IFN-γ. Se ha sugerido que la PSA podría tener un papel relevante en el procesamiento de antígenos ya que es capaz de generar y degradar algunos péptidos antigénicos (2,49). Además, se ha observado un aumento de la expresión de MHC-I en la superficie de las células dendríticas de los ratones $PSA^{-/-}$, aunque la respuesta antiviral mediada por CTLs no se ve afectada (57).

2.1.3.- Endoproteasas citosólicas

Se han descrito varias endopeptidasas que intervienen en el procesamiento de algún ligando de MHC, aunque la mayoría de ellas tienen poco efecto en la expresión global de MHC-I en superficie. Las principales endoproteasas identificadas son:

- Enzima degradora de insulina (IDE): es una metaloproteasa ubicua capaz de degradar distintos sustratos (3,58).

- Oligopeptidasa thimet (TOP): es una metaloproteasa ubicua y la principal responsable de la degradación de los ligandos de MHC-I en los extractos citosólicos (59). La mayoría de los sustratos procesados por esta enzima son péptidos de entre 9 y 17 aminoácidos que recorta hasta los 6-9 residuos. Aunque su principal función en el procesamiento antigénico es degradativa, se ha descrito que está implicada en el recorte de distintos productos generados por el proteasoma (60) y, hasta el momento, se han identificado dos ligandos tumorales que son procesados por esta peptidasa (4).

- Nardilisina: es una metaloproteasa que recorta sustratos con residuos dibásicos (61) y participa en el procesamiento de varios epítopos (4).

- Caspasas: son una familia de cisteinproteasas, principalmente citosólicas, involucradas en procesos de inflamación y apoptosis. La infección por muchos patógenos produce la inducción de la apoptosis celular mediada por la activación de las caspasas (62,63). Se ha descrito que las caspasas 5 y 10 pueden procesar un epítopo de citomegalovirus (5).

Tabla 1.- Resumen de las proteasas implicadas en el procesamiento antigénico.

Proteasa	Abreviatura	Tipo catalítico	Actividad proteolítica	Localización
Proteasoma			Endopeptidasa	Citoplasma
Tripeptidil peptidasa II	TPPII	Serin	Aminopeptidasa	Citoplasma
Dipeptidilpeptidasa 9	DPP9	Serin	Aminopeptidasa	Citoplasma
Bleomicin hidrolasa	BH	Cistein	Aminopeptidasa	Citoplasma
Leucín-aminopeptidasa	LAP	Metalo	Aminopeptidasa	Citoplasma
Aminopeptidasa sensible a puromicina	PSA	Metalo	Aminopeptidasa	Citoplasma
Enzima degradadora de insulina	IDE	Metalo	Endopeptidasa	Citoplasma
Oligopeptidasa thimet	TOP	Metalo	Endopeptidasa	Citoplasma
Nardilisina		Metalo	Endopeptidasa	Citoplasma
Caspasas 5 y 10		Cistein	Endopeptidasas	Citoplasma
Aminopeptidasa asociada al ER	ERAP	Metalo	Aminopeptidasa	ER
Enzima convertasa de angiotensina	ACE	Metalo	Carboxipeptidasa	ER
Peptidasa señal	SP		Endopeptidasa	ER
Peptidasa del péptido señal	SPP	Aspártico	Endopeptidasa	ER y vías vesiculares
Furina		Serin	Endopeptidasa	Trans Golgi
Proprotein convertasa 7	PC7	Serin	Endopeptidasa	Trans Golgi
Catepsinas				Vías vesiculares

2.2.- Transporte de los péptidos al ER por TAP

Los fragmentos peptídicos que no son destruidos en el citosol pueden ser translocados al lumen del retículo endoplásmico (ER) por el transportador asociado con el procesamiento de antígeno (TAP), mediante un proceso dependiente de ATP.

TAP es un heterodímero formado por dos subunidades homólogas denominadas TAP1 y TAP2 (64), que contienen 10 y 9 hélices transmembrana respectivamente, con los dominios de unión a ATP orientados hacia el citosol. Ambas subunidades se unen de forma no-covalente, formando un canal entre ellas que permite la translocación de péptidos al interior del ER (65) (Figura 1B).

TAP une eficientemente péptidos de entre 8 y 16 aminoácidos aunque el tamaño óptimo de los péptidos transportados oscila entre 8 y 12 Aas (66). La eficiencia del transporte también está condicionada por la secuencia del propio péptido, fundamentalmente por los tres residuos amino-terminales y el carboxilo-terminal (67). La presencia del residuo prolina en las posiciones 2 y 3 de un péptido afecta negativamente a su transporte por TAP, tanto en humanos como en ratón (68).

Se han descrito diversas condiciones en las que la función de TAP se encuentra comprometida: en enfermedades genéticas, en células tumorales y en infecciones virales. El Síndrome Linfocitario de Bare tipo I es una enfermedad genética rara debida a un defecto en TAP o en tapasina, en la cual la expresión en la superficie celular de Antígeno Leucocitario Humano de tipo I (HLA-I) se reduce drásticamente (69). Estos pacientes presentan lesiones granulomatosas necrotizantes en la piel e infecciones bacterianas recurrentes, especialmente en el tracto respiratorio. Sorprendentemente estos individuos no muestran una susceptibilidad especial a las infecciones virales aunque la subpoblación de células T $\alpha\beta$, fundamentales para el control de muchas infecciones virales, se encuentra reducida severamente (70). Esta subpoblación de células T, junto con los anticuerpos, las células NK y las T $CD8^+$ $\gamma\delta$ podrían ser suficientes para el control viral en estos individuos. También se ha descrito que en un porcentaje elevado de los tumores existen bajos niveles de expresión de mRNA de TAP1 y/o TAP2. El tratamiento de estas células con IFN-γ permite restaurar los niveles normales de expresión en la mayoría de los casos (71,72). Además, se han identificado diversas proteínas virales capaces de interaccionar con TAP y bloquear su función, mecanismo que dificultaría el reconocimiento de las células infectadas por parte de los CTLs (ver apartado 5).

2.3.- Recorte peptídico en el ER

Los péptidos translocados por TAP pueden ser procesados por las distintas proteasas presentes en el ER.

2.3.1- Las aminopeptidasas del ER

La primera aminopeptidasa residente en el retículo implicada en el procesamiento antigénico fue ERAAP (73) en ratón y su homólogo ERAP1 (74,75) en humanos. Es una metaloproteasa inducible por IFN-γ, responsable del procesamiento de un porcentaje significativo de los ligandos de MHC-I (73-75). ERAP1 recorta preferentemente péptidos de entre 9 y 16 residuos (75,76), lo que coincide con la longitud de los péptidos que son preferentemente transportados por TAP. Esta proteasa recorta eficientemente los sustratos hasta los 8-9 residuos, que es el tamaño óptimo para unirse a MHC-I, a partir de ese tamaño su actividad se bloquea o se ralentiza, lo que no sucede en otras aminopeptidasas (76). La deficiencia de ERAAP en el modelo de ratón afecta significativamente al repertorio peptídico presentado por MHC-I (77-80).

Posteriormente, se identificó en humanos una segunda aminopeptidasa de ER que no existe en ratón, ERAP2 (81,82). La homología de secuencia entre ERAP1 y ERAP2 es del 50% y la especificidad de corte de ambas proteasas es diferente (83). Se ha observado una actividad concertada de ambas enzimas capaz de participar en el procesamiento *in vitro* de ligandos de HLA-I (84,85). Estas enzimas se encuentran en pequeñas cantidades formando un heterodímero ERAP1/2 en el ER (84).

2.3.2.- Carboxipeptidasas

Aunque se había postulado que no existían carboxipeptidasas en el ER implicadas en el procesamiento antigénico (86,87), posteriormente se descubrió la implicación de la enzima convertidora de angiotensina (ACE) en la generación del extremo carboxilo de varios epítopos presentados por MHC-I (88-91). ACE es una metalo-dipeptidasa que además de la angiotensina procesa múltiples sustratos. Pese a que es una ectoenzima de membrana se ha comprobado que posee actividad funcional en el ER (92).

2.4.- Unión de los péptidos a MHC-I

Las proteínas de MHC-I (HLA-I en el ser humano) están codificadas por una familia de genes altamente polimórficos denominados genes del complejo principal de histocompatibilidad. En el genoma humano existen tres *loci* localizados en el brazo corto del cromosoma 6, llamados HLA-A, -B y -C, que codifican para estas proteínas. El elevado polimorfismo de estos genes, supone la existencia de miles de alelos diferentes en cada *locus* (IMGT/HLA Database http://www.ebi.ac.uk/ipd/imgt/hla/stats.html).

El MHC-I está formado por dos cadenas polipeptídicas unidas de forma no covalente: una glicoproteína de unos 45 kDa o cadena pesada y una subunidad de 12 kDa denominada β_2-microglobulina (β_2m) (Figura 2). La cadena pesada consta de tres dominios

extracelulares (α1, α2 y α3) seguidos de un dominio transmembrana, que permite el anclaje a la membrana plasmática, y una cola citoplásmica relativamente corta. Los dos dominios amino-terminales (α1 y α2) se pliegan para formar una estructura compuesta por una lámina de ocho cadenas β antiparalelas sobre las que se asientan dos α-hélices también antiparalelas (93) que conforman una cavidad longitudinal hidrofóbica que puede alojar péptidos de entre 8 y 10 aminoácidos en una conformación extendida y flexible. Este surco de unión de péptidos incluye varias subcavidades, situadas entre la lámina β y las regiones helicoidales, capaces de interaccionar específicamente con las cadenas laterales de determinados aminoácidos denominados residuos de anclaje de los péptidos (94,95). Los residuos localizados en las zonas de unión al péptido son los más polimórficos de toda la molécula (96-98), de tal manera que distintas moléculas de HLA-I presentan sitios de unión de péptidos diferentes, lo que permite que cada alelo de MHC-I presente un repertorio peptídico distinto, con unos motivos de anclaje definidos.

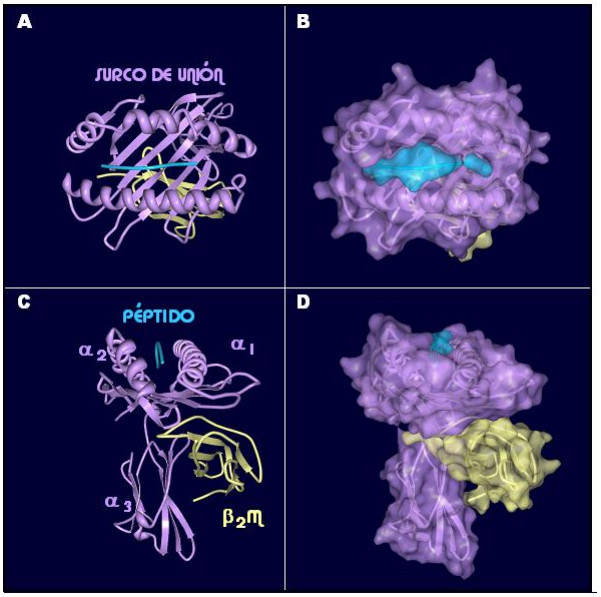

Figura 2.- Estructura tridimensional de la molécula de MHC-I
A y B: Vista apical de los dominios α_1 y α_2 del MHC-I donde se localiza el surco de unión al péptido. C y D: Estructura de la molécula de MHC-I con los tres dominios extracelulares de la cadena pesada y la β_2m.

El dominio α3 se pliega formando una estructura similar a la de los dominios tipo inmunoglobulina. Este segmento contiene un bucle que interacciona con la molécula CD8 del linfocito T. La β_2m presenta también una estructura tipo inmunoglobulina e interacciona principalmente con el dominio α3 de la cadena pesada en todas las moléculas de MHC-I (99).

Para que la β_2m y el péptido puedan unirse a la cadena pesada de MHC-I se requiere una serie de cambios conformacionales previos. La cadena pesada del MHC-I se inserta cotraduccionalmente en la membrana del ER donde se asocia con una chaperona tipo lectina, la calnexina, que la mantiene parcialmente plegada. La unión de la β_2m al dominio α3 produce un cambio conformacional en la molécula de MHC-I que permite el intercambio de la calnexina por otra lectina, la calreticulina. Este trímero se encuentra mayoritariamente formando parte del llamado complejo de carga (100,101), donde se asocia con la tiol oxidoreductasa ERp57, la disulfuro isomerasa (PDI) y la tapasina, que actúa de puente entre el complejo y TAP (Figura 3). Dichas interacciones finalizan con el ensamblaje del péptido al heterodímero MHC-I/β_2m.

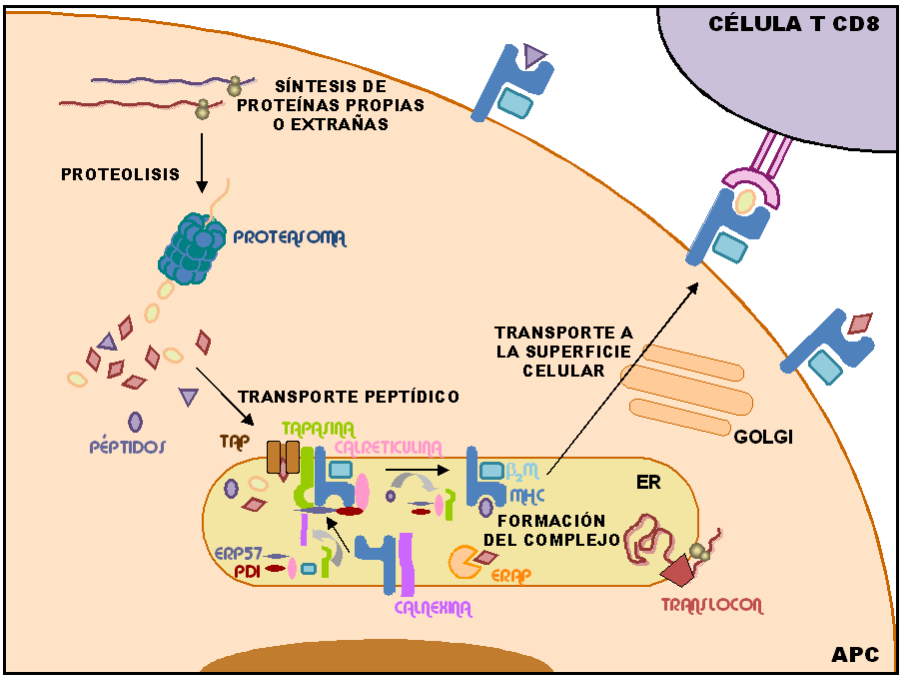

Figura 3.- Presentación MHC
Ruta de procesamiento y presentación de antígenos de MHC-I. Los péptidos generados en el citosol son transportados por TAP al lumen del ER, donde se asocian con la correspondiente molécula de MHC-I y la β_2m. Este proceso requiere la formación previa del complejo de carga formado por TAP, tapasina (verde), calreticulina (rosa), ERp57 (morado) y PDI (rojo). La unión de un ligando de alta afinidad a MHC-I induce su disociación del complejo de carga y el transporte del complejo MHC-I/β_2m/péptido a la superficie celular.

2.5.- Reconocimiento de los péptidos unidos a MHC por los linfocitos T CD8$^+$

El complejo trimolecular estable, MHC-I/β_2m/péptido, viaja a través de la ruta exocítica hasta alcanzar la superficie celular, donde queda expuesto, pudiendo ser reconocido por el receptor de la célula T (TCR) de los CTLs específicos (99).

La interacción del complejo MHC-I/β_2m/péptido con el TCR/CD3 produce la activación del linfocito T (102). Esta interacción requiere la participación de diversas moléculas accesorias como CD8, CD2, CD45, CTLA-4 y CD28, así como de sus respectivos ligandos (103). La activación del CTL conduce además de a la secreción de citoquinas, en donde la interleuquina 2 es el principal factor de crecimiento autocrino, a la proliferación clonal y a la diferenciación de los linfocitos T $CD8^+$.

Los linfocitos T activados pueden destruir células infectadas mediante la liberación de gránulos secretorios que contienen diferentes proteínas como perforinas y granzimas. Las primeras son capaces de inducir la formación de poros en la membrana de la célula diana. Este proceso provoca la pérdida de material citoplásmico y la desestabilización osmótica permitiendo la entrada en la célula de unas serín-proteasas, llamadas granzimas, capaces de activar a las caspasas celulares e induciendo la apoptosis de la célula diana. En los gránulos de los CTLs también se ha encontrado otra proteína, la granulisina, que puede alterar la permeabilidad de las membranas tanto de las células diana como de las microbianas (104). Además, los CTLs activados expresan en su superficie una proteína denominada ligando Fas que puede interaccionar con su receptor específico presente en la membrana de la célula diana, activando a las caspasas e induciendo la apoptosis en la célula diana (105).

La respuesta inmunitaria mediada por los linfocitos T $CD8^+$ frente a antígenos extraños disminuye según transcurre el tiempo desde la inmunización, quedando solo una población de memoria que permite una respuesta rápida y efectiva frente a una reinfección (106). Esto se debe a la apoptosis de la mayoría de los CTLs que se ven privados de sus estímulos de supervivencia al desaparecer el antígeno y las citoquinas generadas por la inmunidad innata.

3.- Vías alternativas de procesamiento antigénico

Aunque la vía clásica de procesamiento antigénico es la principal fuente de péptidos para la formación de complejos de MHC, se han identificado diferentes proteasas y vías de procesamiento alternativas capaces de generar algunos epítopos concretos (Figura 4). A pesar de la actividad de éstas y otras peptidasas (107), cuando se inhibe al proteasoma la expresión de MHC-I en la superficie celular se reduce de forma significativa (108). También se ha observado una importante reducción en ausencia de TAP, ya que el aporte de péptidos desde el citosol queda comprometido, siendo entonces las principales fuentes de aporte de ligandos las vías secretorias y vesiculares y otras vías alternativas que permiten la entrada de péptidos citosólicos al ER.

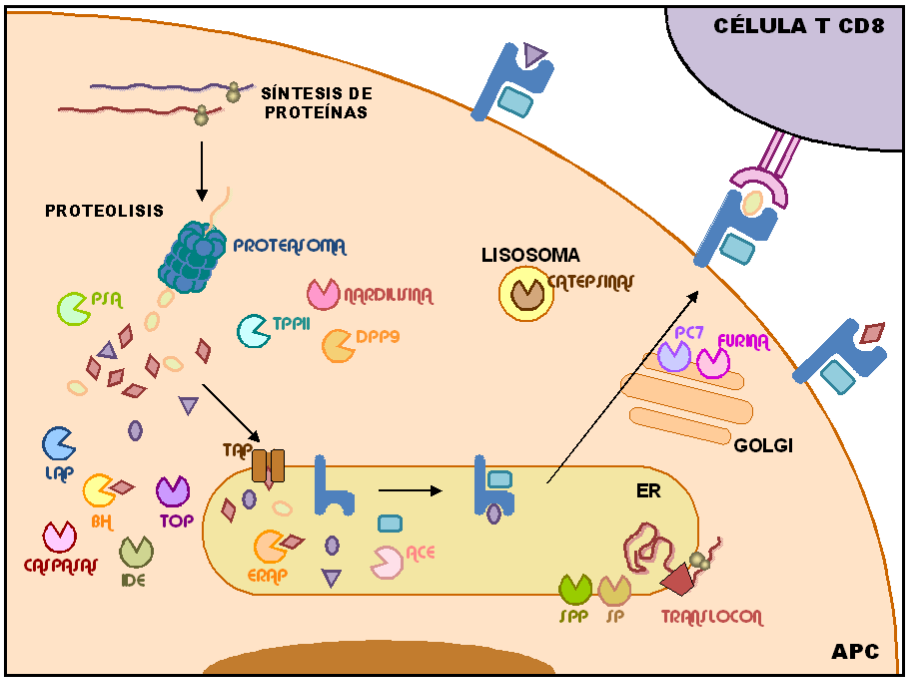

Figura 4.- Proteasas implicadas en el procesamiento antigénico.
Revisión de las peptidasas que intervienen en la generación de epítopos de MHC. Las diferentes proteasas están representadas en función de su actividad proteolítica: endoproteasas (boca del "comecocos" hacia arriba), aminopeptidasas (boca hacia la derecha) y carboxipeptidasas (boca hacia la izquierda).

Los primeros estudios realizados con células deficientes en TAP revelaron que la mayoría de los péptidos provenían de secuencias señal (13,14). En los últimos años se ha incrementado el número de ligandos inmunoprecipitados de células deficientes en TAP y, aunque los ligandos derivados de secuencias señal se han seguido identificando (26), la mayoría provienen de proteínas citosólicas o nucleares, desconociéndose hasta el momento cómo son capaces de acceder a la vía secretoria (109-111).

3.1.- Ligandos independientemente de TAP procedentes de secuencias señal

Las secuencias señal están formadas, generalmente, por tres dominios: una región central hidrofóbica de 6-15 Aas, otra carboxilo terminal polar, con Aas sin carga de pequeño tamaño y una amino terminal con carga neta positiva (112). Las secuencias señal se insertan cotraduccionalmente en la membrana del retículo y son recortadas en su extremo carboxilo por la peptidasa señal (SP) (13,14). Posteriormente, la proteasa del péptido señal (SPP) (15,113) corta la secuencia señal que se encuentra retenida en la membrana, liberando el extremo amino de nuevo al citosol y el carboxilo al lumen del ER, donde podría unirse al MHC-I. El mecanismo por el cual se forma el heterodímero MHC-I/péptido en ausencia de TAP o si el complejo de carga o alguna chaperona están

involucrados en su formación se desconoce, pero se cree que la hidrofobicidad de estos ligandos puede favorecer la interacción.

La SP tiene un papel relevante en la generación del repertorio peptídico de algunos alelos de HLA-I en células deficientes en TAP (26) y de algunos epítopos (13,14,114), aunque también se ha descrito que su actividad puede ser destructiva para otros (115).

La SPP es una aspartilproteasa integral de membrana que ha sido implicada en el procesamiento de tres epítopos, uno dependiente de proteasoma y de TAP (116), otro derivado de la secuencia señal de la calcitonina que es independiente de proteasoma y de TAP (117,118) y otro situado en el extremo carboxilo-terminal de una proteína de transmembrana (119). Además, el silenciamiento de esta peptidasa supone una reducción de la expresión en superficie de MHC-I (119).

3.2.- Ligandos independientes de TAP de proteínas citosólicas

En diversos trabajos se ha observado que algunos de los ligandos de MHC-I presentados independientemente de TAP proceden de proteínas citosólicas y que en su procesamiento está implicado el proteasoma u otras proteasas citosólicas (120,121), por lo que debe existir un modo alternativo al transporte por parte de TAP por el que estos péptidos accedan a compartimentos vesiculares en los que se encuentren moléculas de MHC-I. Se han propuesto diferentes mecanismos de transporte alternativo como la difusión pasiva, el transporte a través del translocon Sec61, el transportador lisosomal (TAPL) (122) u otros transportadores no identificados (120).

3.3.- Ligandos generados en la vía secretoria y vesicular independientemente de TAP

La furina y la PC7 son proproteín-convertasas de la familia de las serín-proteasas, que permiten la maduración de varias proproteínas y procesan polipéptidos celulares y patogénicos (123). Presentan una amplia distribución tisular y se encuentran principalmente en el trans Golgi y en vesículas endocíticas. La especificidad de la furina es muy restrictiva, ya que recorta tras varios residuos básicos con una secuencia previa al corte de RXR/KR, mientras que la PC7 normalmente actúa tras un residuo de arginina. Se ha descrito que la furina procesa varios epítopos en células y ratones deficientes en TAP (16,124,125) y otros ligandos de MHC-I a partir de antígenos exógenos (126,127). Aunque no se han descrito ligandos de HLA procesados por la PC7, se ha observado que es necesaria para el reciclaje de las moléculas de MHC-I que son inestables en la superficie celular a compartimentos post-ER (128). Dado que ninguna de las dos enzimas es activa en el ER, es posible que los péptidos procesados lleguen allí por transporte vesicular

retrógrado (129) o que la unión MHC-I/péptido se produzca en algún compartimento vesicular posterior.

3.4.- Ligandos independientes de TAP procesados en lisosomas

También se han identificado ligandos de MHC-I generados en endolisosomas por la actividad de las catepsinas (17), cisteín o aspártico proteasas localizadas en la vía endocítica donde su principal función es la degradación de proteínas (130). Se han descrito más de una decena de catepsinas con distinta distribución tisular, especificidad de sustrato y función. Aunque están principalmente involucradas en la generación de ligandos de MHC-II, se ha observado que también están implicadas junto con la furina en la generación de un ligando de MHC-I por una vía vacuolar independiente de TAP (17).

Se ha observado que la autofagia puede aumentar el procesamiento de antígenos de MHC-I (131-133), permitiendo la entrada de proteínas citosólicas a compartimentos altamente degradativos en los que también se localiza MHC-I (134).

4.- Poxvirus

Los poxvirus son virus de gran tamaño, entre 200-300 nm de diámetro y de alta complejidad estructural cuyo ciclo infectivo tiene lugar en el citoplasma. Su genoma es DNA de doble cadena, oscila entre 130-375 kpb y codifica para un gran número de proteínas inmunomoduladoras que permiten a estos virus atenuar la respuesta inmune del hospedador (135,136). Los poxvirus que infectan a los vertebrados se dividen en 8 géneros, de ellos solo *Orthopoxvirus* (viruela, vaccinia, monkeypox y cowpox), *Parapoxvirus*, *Molluscipoxvirus* y *Yatapoxvirus* son patogénicos para los humanos, produciendo en general enfermedades exantematosas (137).

4.1.- Los orígenes de la vacunación: la erradicación de la viruela

De todas las pandemias que han asolado a la humanidad, la viruela ha sido la más extendida y mortífera. Desde hace un milenio se ha intentado controlar esta enfermedad mediante la inoculación con pus o costras de enfermos de viruela a individuos sanos (18). Aunque en general, este proceso fue un método eficaz para prevenir la viruela, en algunos casos el virus inoculado era capaz de matar al individuo o de provocar una nueva epidemia. En 1796 el médico inglés Edward Jenner demostró que la inoculación con material, posteriormente identificado como cowpox, procedente de una pústula proveniente de personas que habían estado en contacto con el ganado vacuno protegía frente a la viruela. Dos siglos después, gracias a un programa de vacunación global coordinado por la Organización Mundial de la Salud, se pudo erradicar este peligroso patógeno. El inmunógeno empleado en este programa de vacunación no fue el virus cowpox empleado originalmente, sino otro miembro de la familia de los poxvirus denominado vaccinia.

4.2.- Vaccinia

El virus vaccinia realiza su ciclo infectivo en el citoplasma al igual que el resto de los Poxvirus. Es un virus de DNA de doble cadena de aproximadamente 200 kpb con tres tipos de genes: tempranos, intermedios y tardíos que codifican unas 200 proteínas. Tras la síntesis de las proteínas estructurales tardías, el virión se ensambla recubriéndose de membranas provenientes del compartimento intermedio entre el ER y el aparato de Golgi formando partículas inmaduras (138). Éstas sufren diversos cambios estructurales y bioquímicos dando lugar al virus maduro intracelular, que constituye la mayor parte de las partículas infecciosas. Algunas de estas partículas se rodean de membranas de las cisternas de la red del trans-Golgi, dando lugar a los virus intracelulares con envuelta (139), que son transportados a la superficie de la célula mediante el citoesqueleto. Una vez allí, la membrana externa del virus se fusiona con la celular, liberándose los virus extracelulares con envuelta al exterior.

Desde hace varias décadas vaccinia y otros poxvirus han sido empleados como vectores para la expresión de distintas proteínas pertenecientes a diversos virus patogénicos, ya que permiten fácilmente la incorporación de genes adicionales con niveles de expresión elevados. Uno de los primeros estudios que permitió obtener respuesta contra una proteína de otro virus incorporada al genoma de vaccinia fue el de Cox et al. en el que obtenían CTLs contra la glicoproteína de la envuelta del virus de la inmunodeficiencia humana (HIV) (140).

5.- Evasión viral frente a la respuesta inmune celular

La presión selectiva por parte del sistema inmune ha permitido la evolución de distintos mecanismos de evasión que afecta tanto a la respuesta innata como a la adaptativa (141). Los linfocitos T citotóxicos juegan un papel clave en la eliminación de las células infectadas, por ello muchas de las estrategias de evasión viral van encaminadas a dificultar su actividad mediante la reducción de la expresión de MHC-I en la superficie celular. Los herpesvirus y los poxvirus son capaces de acelerar la degradación o la endocitosis del MHC-I, de interferir en el reconocimiento por el TCR o incluso de bloquear distintas proteínas implicadas en el procesamiento antigénico (141).

Una etapa crítica en el procesamiento antigénico es el transporte de los péptidos desde el citosol al ER por TAP de tal manera que se han identificado cuatro genes de herpesvirus que codifican para proteínas que interaccionan con TAP, inhibiendo su función mediante distintas estrategias (Figura 5) (41,141,142):

- impidiendo la unión de los péptidos al transportador, como la proteína ICP-47 del virus del herpes simple (HSV) -1 y -2, que actúa como un pseudosustrato de TAP,

- interfiriendo la unión de ATP a TAP, como la proteína US6 de citomegalovirus (CMV),

- evitando los cambios de conformación que requiere TAP para transportar a los péptidos, como actúa la proteína UL49.5 del virus de la varicela. La proteína homóloga en el virus del herpes bovino (BHV) también induce la degradación de TAP por parte del proteasoma.

El cuarto gen, que se encuentra en el virus de Epstein-Barr (EBV), codifica para una proteína, la BNLF2a, que combina las dos primeras estrategias para inhibir TAP.

Además, en el año 2009 se identificó al primer virus, no perteneciente a la familia de herpesvirus, capaz de bloquear TAP: cowpox. La responsable de esta inhibición es una proteína de membrana tipo II, la CPXV12, capaz de bloquear la unión de ATP a TAP (21,143,144).

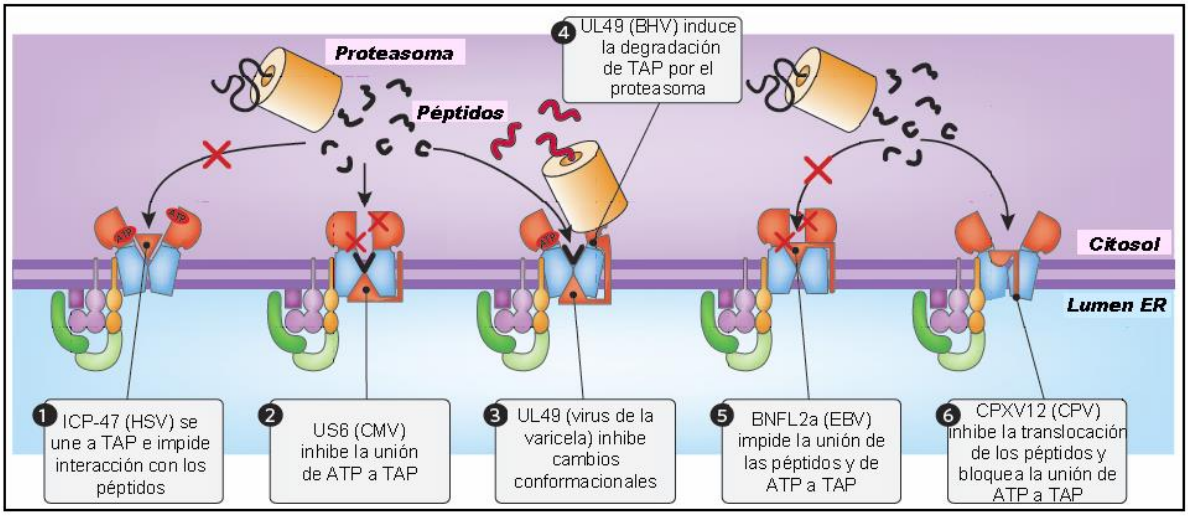

Figura 5.- Inhibición de TAP por proteínas virales como medio de evasión inmune.
Se representan las distintas proteínas virales capaces de inhibir el transporte de péptidos al ER mediado por TAP y el mecanismo empleado para ello. CPV se refiere al virus cowpox. Imagen adaptada a partir de (41)

6.- Espectrometría de masas e inmunoproteómica

La espectrometría de masas es una técnica que permite producir aniones en fase gaseosa a partir de moléculas orgánicas o inorgánicas, acelerarlos y separarlos en función de su relación masa-carga (m/z), mediante el uso de un campo magnético homogéneo. Esta técnica permite determinar el peso molecular y la abundancia de los componentes de una mezcla. Los iones identificados pueden ser moléculas enteras, aductos o fragmentos de moléculas. El análisis de estos últimos permite obtener información sobre la estructura química del compuesto del que derivan. Por todo ello, esta técnica es muy útil para el

estudio de mezclas peptídicas complejas como las que se obtienen de la inmunoprecipitación de los ligandos de MHC-I.

Desde que en 1992 se identificaron, mediante el empleo de la espectrometría de masas, las primeras secuencias de péptidos eluidos junto a la molécula HLA-A*02 (145), el número de ligandos de MHC descubiertos mediante esta tecnología no ha dejado de aumentar, de tal manera que en la actualidad por cada alelo de MHC-I analizado se pueden identificar miles de péptidos diferentes, con secuencias y longitudes distintas. Dado que el número de ligandos endógenos procedentes de proteínas celulares es mucho mayor que el de los ligandos de proteínas del patógeno, estos estudios requieren de una separación previa de los péptidos por HPLC y una comparación exhaustiva de los espectros de fragmentación obtenidos a partir de células infectadas y no infectadas para poder identificar los péptidos provenientes del patógeno. La mejora de la sensibilidad de los espectrómetros de masas y el desarrollo de las herramientas bioinformáticas aplicadas a la resolución de los espectros de fragmentación (MS/MS) ha permitido que el número de ligandos identificados para un mismo alelo pasara de decenas a varios miles por ensayo (146). Este tipo de estudios han puesto de manifiesto la correlación existente entre la secuencia de los péptidos inmunoprecipitados y el polimorfismo alélico (147). Además, las secuencias identificadas mediante esta tecnología han contribuido a la identificación de los motivos de anclaje de múltiples moléculas de MHC-I y a la generación de herramientas de predicción de ligandos de MHC-I como SYFPEITHI (http://www.syfpeithi.de), BIMAS (http://www-bimas.cit.nih.gov), etc.

V.-OBJETIVOS

El objetivo principal de este trabajo ha sido la identificación de los ligandos HLA-I, tanto virales como endógenos, generados en células deficientes en TAP y el posterior análisis de las vías proteolíticas que contribuyen al procesamiento de estos ligandos. Para la consecución de este objetivo se ha llevado a cabo:

- El análisis mediante técnicas inmunoproteómicas de los ligandos virales y endógenos presentados por HLA-I en células deficientes en TAP.
- La evaluación de la respuesta de linfocitos T $CD8^+$ frente a estos ligandos virales en modelos de ratones transgénicos para el correspondiente alelo de HLA-I.
- El estudio de las vías de procesamiento implicadas en la síntesis de los ligandos identificados.

VI.-ARTÍCULOS

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Diversity of Natural Self-Derived Ligands Presented by Different HLA Class I Molecules in Transporter Antigen Processing-Deficient Cells

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Abstract

The transporter associated with antigen processing (TAP) translocates the cytosol-derived proteolytic peptides to the endoplasmic reticulum lumen where they complex with nascent human leukocyte antigen (HLA) class I molecules. Non-functional TAP complexes and viral or tumoral blocking of these transporters leads to reduced HLA class I surface expression and a drastic change in the available peptide repertoire. Using mass spectrometry to analyze complex human leukocyte antigen HLA-bound peptide pools isolated from large numbers of TAP-deficient cells, we identified 334 TAP-independent ligands naturally presented by four different HLA-A, -B, and -C class I molecules with very different TAP dependency from the same cell line. The repertoire of TAP-independent peptides examined favored increased peptide lengths and a lack of strict binding motifs for all four HLA class I molecules studied. The TAP-independent peptidome arose from 182 parental proteins, the majority of which yielded one HLA ligand. In contrast, TAP-independent antigen processing of very few cellular proteins generated multiple HLA ligands. Comparison between TAP-independent peptidome and proteome of several subcellular locations suggests that the secretory vesicle-like organelles could be a relevant source of parental proteins for TAP-independent HLA ligands. Finally, a predominant endoproteolytic peptidase specificity for Arg/Lys or Leu/Phe residues in the P_1 position of the scissile bond was found for the TAP-independent ligands. These data draw a new and intricate picture of TAP-independent pathways.

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Introduction

Proteolysis, by the proteasome and other cytosolic proteases, of both newly synthesized proteins and the mature cell proteome continuously generates short peptides that are transported into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP) [1]. These peptides are assembled with a nascent HLA class I heavy chain and β2-microglobulin to generate stable HLA/peptide complexes that are exported to the cell membrane and subjected to cytotoxic $CD8^+$ T lymphocyte recognition (reviewed in [2]).

Non-functional TAP complexes, which can be produced by mutations in the TAP gene, have been described in both humans [3] and mice [4]. Patients with an HLA class I deficiency have a reduced functional $CD8^+$ population but may appear asymptomatic for long periods of time with only a limited susceptibility to chronic respiratory bacterial infections. Thus, their immune systems must be reasonably efficient, and in addition to different unaltered layered defenses, it remains possible that the reduced cytolytic $CD8^+$ αβ T subpopulation that is specific for TAP-independent antigens may contribute to immune defenses that protect against severe infections in these individuals.

Although TAP-independent viral epitopes are known (reviewed in [5–7]), few studies have analyzed the cellular TAP-independent HLA class I peptide repertoire. TAP-deficient cells have been described as having very limited antigen processing capacity [8], with predominant proteolytic ER signal peptidase (SPase) activity [9]. Therefore, is the TAP-independent HLA peptidome so limited in TAP-deficient cells, as suggested by these studies? The identification of self-derived ligands presented in the same cells by several common HLA antigens with very different TAP dependency is of major interest. Therefore, using a *high-throughput* immunopeptidomics analysis, we analyzed the TAP-independent HLA peptidome isolated from large numbers of TAP-deficient cells and bound to different HLA alleles. In this study, we identified more than three hundred TAP-independent ligands bound to different HLA-A, -B, and -C class I molecules.

Materials and Methods

Cell Lines

T2 is a human T cell leukemia/B cell line hybridoma cell line that has a large homozygous deletion within the MHC, including both TAP genes and all of the functional class II genes [10–12]. In addition, this cell line expresses low levels of HLA class I molecules on the cell surface [13]. T2 cells transfected with B*2705 have been previously described [14]. Transfected RMA-S tumour of T cells (deficient in TAP that expresses low levels of cell surface MHC class I) expressing HLA-B*2705 have also been previously described [15]. All cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 5 μM β-mercaptoethanol.

Synthetic Peptides

Peptides were synthesized in a peptide synthesizer (model 433A; Applied Biosystems, Foster City, CA) and purified by reversed-phase HPLC. The correct molecular mass of each peptide was established with a Reflex IV MALDI-TOF instrument (Brucker-Franzen Analytik, Bremen, Germany), and their correct composition was determined with a Deca XP LCQ mass spectrometer (Thermo Fisher, San Jose, CA).

Isolation of HLA-bound Peptides

HLA-bound peptides were isolated from 4 x 10^{10} healthy T2-B27 transfected cells or vaccinia (VACV)-WR-infected T2-B27 transfected cells. Cells were lysed at 4°C in 1% CHAPS (Sigma), 20 mM Tris/HCl buffer, and 150 mM NaCl, pH 7.5, in the presence of a protease inhibitor cocktail [16,17]. First of all, the soluble fraction of cell extracts was treated with immunoaffinity columns without antibody to discard unspecific binding of peptides. Next, HLA-peptide complexes were isolated via affinity chromatography of the soluble fraction of cell extracts with the following mAbs, used sequentially: PA2.1 (anti-HLA-A2) [18], ME1 (anti-HLA-B27) [19], and W6/32 (specific for a monomorphic HLA class I determinant) [20] (Figure S1). HLA-bound peptides were eluted at room temperature with 0.1% aqueous trifluoroacetic acid (TFA), separated from the large subunits and concentrated with a Centricon 3 ultrafiltration device (Amicon, Beverly, MA) exactly as previously described [16,17].

Electrospray-Orbitrap Mass Spectrometry Analysis

Peptide mixtures recovered after the ultra-filtration step were concentrated with Micro-Tip reverse-phase columns (C_{18}, 200 μl, Harvard Apparatus, Holliston, MA) [16]. Each C_{18} tip was equilibrated with 80% acetonitrile in 0.1% TFA, washed with 0.1% TFA, and then loaded with the peptide mixture. The tip was then washed with an additional volume of 0.1% TFA and the peptides were eluted in 300 μl with 80% acetonitrile in 0.1% TFA. Peptide samples were then concentrated to approximately 18 μl using vacuum centrifugation [16,17].

HLA class I peptides that had been immunoprecipitated with each HLA-specific mAb were analyzed by μLC-MS/MS using an Orbitrap XL mass spectrometer (Thermo Fisher) fitted with a capillary HPLC (Eksigent, Dublin, CA) [16,17]. The peptides were resolved on homemade Reprosil C18 capillary columns (75 micron ID) [21] with a 7%–40% acetonitrile gradient for 2 h in the presence of 0.1% formic acid. The seven most intense masses that exhibited single-, double-, and triple-charge states were selected for fragmentation from each full mass spectrum by collision-induced dissociation.

Database Searches

Raw mass spectrometry data were analyzed using various software tools: Proteome Discoverer 1.0 SP1 (Thermo-Fisher) combining the results of Sequest 3.31 and Bioworks Browser 3.3.1 SP1 (Thermo-Fisher) [22], using the human part of the NCBI database (Feb 2012) including 729,880 proteins. The search was not limited by enzymatic specificity, the peptide tolerance was set to 0.01 Da, and the fragment ion tolerance was set to 0.5 Da [16,17]. Oxidized methionine was searched as a variable modification. Other search criteria were set such that the search was not limited by any methodological bias (selection of individual protein, use of HLA consensus scoring algorithms, etc.). To exclude peptides that could contaminate the peptide pool, a search using the bovine part of the NCBI database (Feb 2012) including 29,925 proteins was performed. No bovine serum peptides were identified bound to HLA class I molecules.

Identified peptides were selected if the following criteria were met: mass accuracy $\leq$0.005 Da or $<$5 ppm; Sequest Xcorr $>$1.5 for singly, $>$2.5 for doubly, and $>$3.5 for triply charged peptides; ΔCn $>$0.1; Proteome Discovered P score $>$20; and P(pep) $<1\times10^{-2}$ with Bioworks Browser [16,17]. When the MS/MS spectra fitted more than one peptide, only the highest scoring peptide was selected. The false-positive rate for peptide identification yielded a 2% based on a search of a reversed database, wherein the amino acid sequences of all proteins are reversed. In addition, two synthetic peptides were made, and their MS/MS spectra were used to confirm the assigned sequences (Figures S2 and S3).

MHC/Peptide Stability Assays

The following synthetic peptides were used as controls in complex stability assays: Flu NP (SRYWAIRTR, HLA-B27-restricted) [23], RSV $M_{76–84}$ (SRSALLAQM, HLA-B27-restricted) [16], and C4CON (QYDDAVYLK, HLA-Cw4-restricted) [24]. RMA-S B*2705 transfectant cells were incubated at 26°C for 16 h in RPMI 1640 medium supplemented with 10% heat-inactivated FBS. This allows the expression of empty MHC class I molecules (without antigenic peptide) at the cellular membrane that are stable at 26°C but not at 37°C. The cells were washed and incubated for 2 h at 26°C with various concentrations of peptide in the same medium without FBS. The cells were maintained at 37°C for an additional 4 h and then collected for flow cytometry. This method allows empty MHC class I molecules to become internalized and can thus discriminate between bound and unbound peptides. MHC expression was measured using 100 μl of hybridoma culture supernatant containing ME1 (anti-HLA-B27) mAb as previously described [25]. Samples were acquired on a FACSCanto flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed using CellQuest Pro 2.0 software (BD Bioscience). Cells incubated without peptide had peak fluorescence intensities close to background staining with secondary Ab alone. The fluorescence index was calculated at each time point as the ratio of the mean channel fluorescence of the sample to that of the control incubated without peptide. The data are mean values of the three experiments.

Results

Physiological Processing Generates Multiple Cellular Ligands Bound to HLA Alleles with Distinct TAP Dependency in the Same Human TAP-deficient Cell Line

To date, approximately 70 human TAP-independent ligands from classical HLA class I molecules are known [7,9], and are mostly restricted to HLA-A2 and derived by cleavage of signal

sequences generated by the SPase complex. Thus, the comparison of TAP-independent peptide pools derived from HLA-A2, HLA-B27, an allele high TAP-dependent [26], and other HLA class molecules, such as HLA-B51 or -Cw1, with no data about their TAP dependency, could be relevant in the study of alternative antigen processing pathways.

In a previous study, HLA-A2, -B27, -B51, and -Cw1-bound peptide pools were isolated from large amounts of either healthy or the vaccinia (VACV)-infected human TAP-deficient cell line (Figure S1) and eleven VACV viral ligands were identified [17]. Moreover, the use of several software tools in these same samples over a human proteome database resolved 111, 77, and 192 fragmentation spectra as peptidic sequences of different human cellular proteins bound to HLA-A2 (Table S1), -B27 (Table S2), and -B51 or -Cw1 (Table S3), respectively. Two different peptide sequences were selected as additional controls for assignment. Both the experimentally detected and the corresponding synthetic peptide MS/MS spectra were identical (Figures S2 and S3). To confirm that HLA-B27 is the MHC class I molecule that presents these ligands, MHC/peptide complex stability assays were performed using TAP-deficient RMA-S cells transfected with the HLA-B27 molecule (Fig. S4).

Collectively, these results indicate that a similar broad range of TAP-independent ligands was endogenously processed and presented by different HLA class I molecules in the same infected cells, despite their differences in TAP dependency [26].

Structural Features of TAP-independent HLA Ligands

HLA-A2, -B51, and -Cw1 class I molecules usually bind peptides approximately 9–11 residues long (SYFPEITHI database: http://www.syfpeithi.de [27]), whereas HLA-B27 could accommodate peptides up to 13–14 residues in a bulged conformation (SYFPEITHI database, [28]). The analysis of size indicated that approximately 60% of the TAP-independent HLA-A2, -B51, or -Cw1 ligands and 40% of the -B27 ligands are longer than those identified in TAP-sufficient cells (Fig. S5). We next studied the anchor motif requirements of these ligands. The HLA-A2, -B51, and -Cw1 alleles present peptides with partially similar anchor motifs (SYFPEITHI database). The classical position 2 anchor motifs are as follows: for HLA-A2 binding, Leu or Met; for HLA-B51, Pro and Ala; and for HLA-Cw1, Ala and Leu. Aliphatic CΩ residues (SYFPEITHI database) are common between these three HLA alleles. For HLA-B27, the anchor motif consists of Arg or Gln at P2 and basic or aliphatic CΩ residues (SYFPEITHI database, [28]). However, the respective anchor motifs were absent in 60–70% of the TAP-independent HLA ligands (Table 1). Likewise, the amino acid preference at the CΩ position was studied and revealed major discrepancies among the HLA-A2, -B51 and -Cw1 ligands of TAP-sufficient versus TAP-deficient cells, although no differences in HLA-B27 ligands were found (Table 1). These data suggest that the relative contribution of the CΩ pocket to the stabilization of unusual peptides differs among these HLA class I alleles.

It is well documented that HLA-A2 binds signal sequence-derived peptides generated by the cleavage of signal sequences from the parental polypeptide by the signal peptidase (SPase) complex [9]. We found that 11% of peptides that bound to the HLA-A2 molecule were derived from the signal sequence of various proteins (Table 2). In addition, 3% of HLA-B27 and -B51 or -Cw1 ligands are located in the region generated by SPase activity (Table 2). Most importantly, a significant fraction of bound ligands identified from HLA-A2 (17%)-, -B27 (23%)- and -B51 or -Cw1 (24%)-associated repertoires are located at the C-terminal position of their respective proteins. Thus, only one endoproteolytic cleavage was needed to release these particular ligands. In contrast, the remainder of peptides required two endoproteolytic cleavages for their generation. Unexpectedly, a similar fraction of the double cleaved HLA ligands were nested set peptides with an identical core but with N- and/or C-extended residues from the same protein (Table 2 and Tables S1, S2, and S3). Representative nested set peptides are depicted in Figure S6.

TAP-independent Processing Generates Multiple HLA Ligands from the same Cellular Proteins

In addition to N- and C-extended peptides (Tables S1, S2 and S3, and Figure S6), some proteins contributing different clustered HLA ligands were identified by mass spectrometry (Tables S1, S2, S3, and S4). Figure 1 depicts a representative example. In the first 80 residues of myosin heavy polypeptide 9 protein, different adjacent or superimposed HLA ligands were processed. In addition, other N- and C-terminal extended peptides that were identified as binding to different HLA class I molecules were identified from the same protein (Figure 1, panel B). Thus, these data indicate that extensive processing of N- and C-terminal regions of some proteins occurs via TAP-independent antigen processing pathways. These recurrent endoproteolytic activities were not restricted based on their location within the polypeptide because several HLA ligands were identified that constitute internal regions of some proteins (Figure S7 and Table S4).

In total, one-fifth of the identified proteins were processed, and their ligands were later presented by two different HLA class I molecules. Approximately 6% of the identified proteins generated multiple TAP-independent ligands associated with HLA-A2, -B27, and -B51 or –Cw1 class I molecules (Table 3 and Tables S4 and S5).

Collectively, these data indicate that some proteins could be widely processed by proteases via TAP-independent pathways, yielding multiple peptides that could be presented by different HLA class I molecules. Thus, these data suggest that greater proteolytic processing occurs than previously supposed in TAP-independent antigen processing pathways.

Cellular Location of Parental Proteins for TAP-independent HLA Ligands

The 334 TAP-independent ligands identified by mass spectrometry arose from 182 parental proteins. Table 4 shows the predicted organelle location of these proteins based on the Gene Ontology database (http://www.geneontology.org) [29]. One-third of the parental proteins were from the ER (8%), Golgi (4%), plasma membrane (14%), and secretory granules (6%), which likely represents their direct processing from transported proteins by resident proteases in the HLA-loading compartments. Strikingly, approximately two-thirds of the parental proteins were from non-secretory subcellular compartments, such as the nucleus (32%), cytoplasm (22%), cytoskeleton (9%), and mitochondria (5%). As such, their respective proteins and/or processed ligands must be transported into the ER lumen by a yet unknown pathway to yield TAP-independent antigen presentation.

The Secretory Vesicle-like Organelle could be an Important Source of Parental Proteins for HLA TAP-independent Ligands

Various TAP-independent ligands from several lysosomal proteins (e.g., lysosomal multispanning membrane protein 5, Figure S6) were identified. Thus, an attractive hypothesis arose that pointed to protein digestion within this degradative organelle as the source of most of the parental proteins yielding the TAP-

Table 1. Amino acid preference at anchor motif P2 and CΩ positions in TAP-dependent versus TAP-independent ligands.

Position	Residue	HLA-A2		Residue	HLA-B27		Residue	HLA-B51, Cw1	
		TAP^{+a}	TAP^{-b}		TAP^{+c}	TAP^{-d}		TAP^{+e}	TAP^{-f}
P2	L/M	73	42	R/Q	100	28	L/A/P	81	39
CΩ	L/V/I/A	92	41	R/F/K/L/Y	91	78	V/I/L	83	31

[a]424 HLA-A2 ligands from SYFPEITHI database.
[b]111 HLA-A2 TAP-independent ligands, see Table S1.
[c]571 HLA-B27 ligands [28].
[d]77 HLA-B27 TAP-independent ligands, see Table S2.
[e]68 HLA-B51 and 9 HLA-Cw1 ligands from SYFPEITHI database.
[f]192 HLA-B51 and -Cw1TAP-independent ligands, see Table S3.
[g]Data are expressed in percentage.
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independent ligands identified by mass spectrometry. Thus, the distribution of both the source proteins of the TAP-independent peptidome and those of the lysosomes previously published [30,31] was compared. Table 4 shows a very different pattern of organelle location of constitutive proteins between this subcellular organelle and the TAP-independent peptidome. Thus, the lysosomes are not likely to be the primary source of peptides in the TAP-independent peptidome. As some lysosomal proteins are expressed in other secretory organelles, and because diverse vesicular organelles harbor different organelle-specific constitutive proteins, the composition of several organelles was analyzed. Only secretory vesicles from human neutrophils [32], similar to those identified in several immune cells including T and B cells) [33,34], have shown a similar predominance of cytoplasm, nucleus and plasma membrane proteins in the ratios observed for the TAP-independent peptidome (Table 4). Further, 51% of the parental proteins identified as part of the TAP-independent peptidome (Table S6) were previously found in the proteome of this secretory vesicle [32]. Thus, secretory-like vesicles could be a relevant source of the parental proteins observed during TAP-independent antigen processing.

Cleavage Specificity of Peptidases Over TAP-independent Ligands

Next, to study the specificity of peptidases involved in the generation of ligands in TAP-defective cells, an analysis was carried out that used mass spectrometry to detect residues on both sides of the hydrolyzed bonds of HLA ligands. When several nested peptides were found, amino and/or carboxyl peptidase activities could be assumed. Accordingly, only the higher possible HLA ligand was examined. Under this hypothesis, 264 TAP-independent ligands (the 79% of peptidome) were analyzed. Figure 2 shows the distribution of amino acids found in the immediate flanking positions of scissile bonds (P_1 or P'_1 residues). The relative abundance of most amino acids was similar when P'_1, but not P_1, residues of HLA ligands were analyzed (panel B versus C). This was also true when the analysis was performed on each individual HLA class I molecule studied (data not shown). Additionally, no correlation was found when similar analyses of P_2, P_3, P'_2, and P'_3 positions were performed (data not shown). In summary, these results indicate that proteases with specificity to some residues in P_1, but not other positions, make the endoproteolytic cleavages to generate TAP-independent ligands. Only four amino acids (Arg, Leu, Lys, and Phe) are increased in the P_1 positions of scissile bonds, which accounted for up to 10% of the total cleavages detected (Fig. 2, panel B). These four major P_1 residues could be processed by different proteases or may represent the specificities of one or a few proteases that generate both P_1 cleavage positions. To resolve these questions, an analysis of the correlation between these four specific amino acid residues and the opposite P_1 N- or C-end residues of HLA ligands was carried out. When Arg was the P_1 C-end of an HLA ligand identified by mass spectrometry, only two amino acid residues (Arg and Lys) were predominantly located in the corresponding P_1 N-end position (Fig. 3B, white boxes). This was true also for the reverse situation; when Arg was the P_1 N-end position, the amino acid residues predominantly located in the P_1 C-end position were also Arg and Lys (Fig. 3B, black boxes). Further, an identical correspondence in the analysis of Lys residue was found with both the P_1 N- and C-end positions (Fig. 3C). Similar analyses with Phe P_1 cleavages have shown that only Phe and Leu residues were mainly located in the equivalent P_1 N- or C-end positions of scissile bonds (Fig. 3D). A minor correlation with the Leu cleavage analysis was found, although Phe and Leu remained as the most abundantly detected residues (Fig. 3E). As several endoproteolytic peptidases have specificity for Arg/Lys or Leu/Phe in the P_1 position of the scissile bond (MEROPS database: http://merops.sanger.ac.uk [35]), the enzymatic activity of only two of these types of peptidases could explain more than half of the identified cleavages derived from the detected TAP-independent ligands (Table S7).

Table 2. Major features of TAP-independent HLA ligands.

Type of peptide	HLA-A2	HLA-B27	HLA-B51, -Cw1
Signal sequence[a]	11[b]	3	3
C-terminal[c]	17	23	24
N-extended[d]	10	18	11
C-extended[e]	4	8	4
N- and C-extended	1	0	1
Total of extended	15	26	16

[a]Peptides located into signal sequence of respective protein.
[b]Data are expressed in percentage of total TAP-independent ligands.
[c]Peptides located in C-terminal position of respective protein.
[d]N-extended peptides respect the minimal ligand identified with identical core.
[e]C-extended peptides respect the minimal ligand identified with identical core.
doi:10.1371/journal.pone.0059118.t002

Low Hydrophobicity in TAP-independent Virus Ligands

In cells infected with the Epstein-Barr virus (EBV), only peptides with high hydrophobicity from the BRLF1 and LMP2 proteins

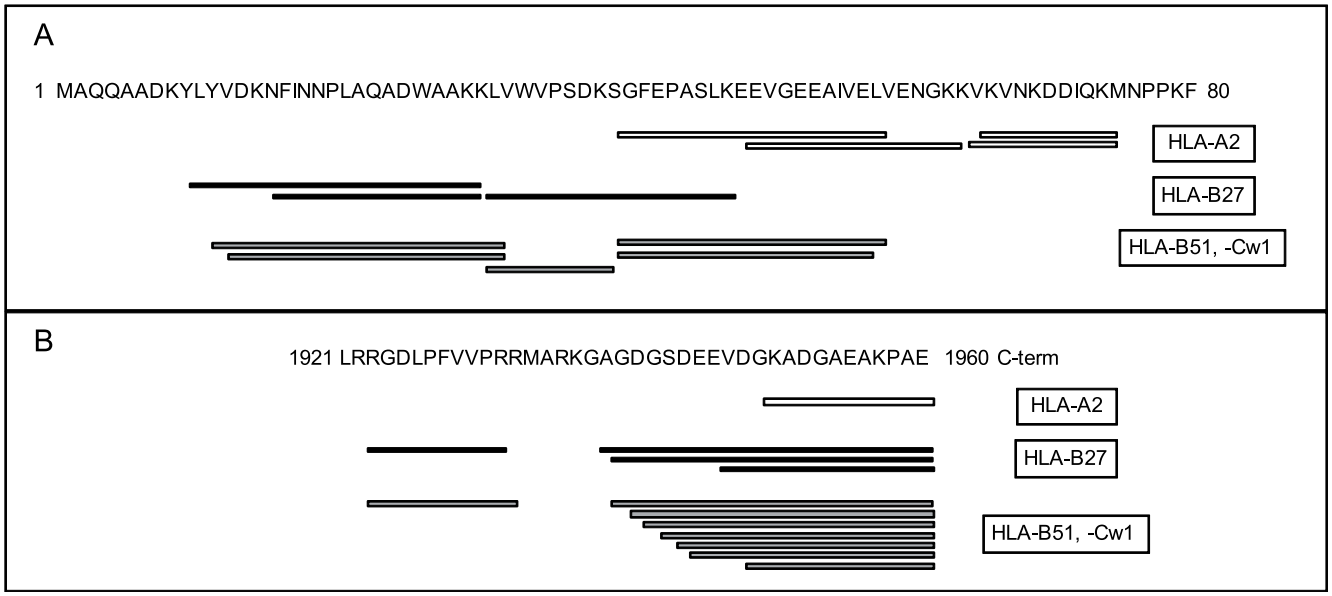

Figure 1. Naturally processed peptides from myosin heavy polypeptide 9 identified by mass spectrometry. Diagram of identified ligands bound to HLA class I molecules in the first 80 (panel A) or last 40 (panel B) residues from myosin heavy chain 9 protein. Ligands specific for HLA-A2 (white boxes), -B27 (black boxes), and –B51 or –Cw1 (gray boxes) are depicted in the lower section of each panel.
doi:10.1371/journal.pone.0059118.g001

were processed by TAP-independent pathways [36,37]. In contrast, hydrophobicity is not a necessary condition for the TAP-independent presentation of vaccinia virus ligands [17]. Because the total number of viral ligands in both studies was low (eight and eleven ligands from EBV or vaccinia virus, respectively), the study of the hydrophobicity of more abundant cellular TAP-independent ligands could be important. The grand average of hydropathicity (GRAVY) (ProtParam tool, ExPASy Proteomics Server, http://www.expasy.ch) mean of approximately 1,000 HLA-A2, -B27, -B51, and -Cw1 TAP-dependent ligands previously described (Table 1) was only 0.0 ± 1.0, and significant differences between the different HLA alleles studied were not found (data not shown), indicating that no hydrophobicity of these HLA ligands exists. A very similar GRAVY measurement (-0.3 ± 0.8) was found for the 337 TAP-independent ligands described in this report. These results show that hydrophobicity is not a necessary condition for the overall TAP-independent presentation of cellular ligands.

Discussion

Identification of self-derived HLA ligands by mass spectrometry analysis contributes to a better understanding of the mechanisms of antigen presentation that are associated with the cellular immune response. While several hundred peptides bound to specific MHC class I alleles are identified in any given immunoproteomics analysis from TAP-sufficient cells, only some tens have been described for TAP-independent HLA ligands from the still very limited number of immunoproteomics studies from TAP-deficient cells. In the first study, 22 and 27 cellular peptides bound to the murine H-2K^d class I molecule were immunoprecipitated from human and mouse TAP-deficient cells respectively [8]. Later, 50 HLA-A2 or -B51 ligands were presented TAP-independently in a human TAP-deficient cell line [9]. Currently, using a sequential immunoprecipitation of several HLA class I molecules, we identified several hundred TAP-independent ligands that were processed and presented by the four class I molecules expressed in the same cell population**.** Thus, the HLA peptidome generated by TAP-independent antigen processing pathways is more diverse than previously assumed. In agreement

Table 3. HLA restriction and number of proteins from TAP-independent ligands.

Number of HLA alleles	% of Proteins[a]	% of TAP-independent ligands[a]
One	75	45
Two	19	22
HLA-A2 and –B27	2	2
HLA-A2 and –B51 or –Cw1	11	10
HLA-B27 and –B51 or –Cw1	6	10
Three	6[b]	33

[a]of the total shown in Tables S1, S2, and S3.
[b]see Table S5.
doi:10.1371/journal.pone.0059118.t003

Table 4. Distribution of proteins by cell location.

Location	TAP$^-$ peptidome	Lysosomes[a]	Secretory vesicles[b]
Cytoplasm	22[c]	16	23
Cytoskeleton	9	0	8
Endoplasmic reticulum	8	2	4
Extracellular	0	0	5
Golgi	4	1	2
Mitochondria	5	2	6
Nucleus	32	5	22
Plasma membrane	14	2	24
Secretory granule[d]	6	72	8

[a]from human T cells [30,31].
[b]from human neutrophils [32].
[c]data are expressed in percentage of proteins listed by cell location based on gene ontology analysis (http://www.geneontology.org) [29].
[d]Secretory granule are represented by melanosomes, lysosomes, platelet granules, endosomes, synaptosomes, exosomes or cytolytic granules as defined in references [30,31].
doi:10.1371/journal.pone.0059118.t004

with previous studies [8,9], the major features of the peptide repertoire bound to classical MHC class I from TAP-deficient cells revealed increased peptide lengths and a lack of strict binding motifs in all HLA class I molecules studied in the current report. The cytosol is a very degradative compartment, having multiple endoproteases and exopeptidases [1]. In contrast, both the vesicular compartments and the secretory pathway (source of TAP-independent ligands) are clearly less degradative [38]. Thus, the absence of high affinity ligands, generated by the TAP-dependent antigen processing, allows the interaction between the high amounts of empty HLA class I molecules and the long TAP-independent ligands with low affinity from TAP-deficient cells [8,39]. In addition, in a previous study using the same HLA peptidome, several vaccinia low affinity ligands were identified that did not conform to the normal anchor motifs used by HLA-A2, -B27 and –Cw1 class I molecules [17]. Nonetheless, the identified HLA-A2 low-affinity ligand generated long-term CTL memory responses against vaccinia virus, even in an HLA-A2 transgenic TAP$^+$ mouse model [17]. These data and other studies [40,41] indicate that low-affinity ligands, such as the self-derived peptides identified in the current report, have functional relevance.

Immunoproteomics analysis of the MHC class II peptidome usually shows ligands represented by several length variants of the same core, thereby forming sets of nested peptides varying by several residues at the N- or C-terminal ends (summarized in SYFPEITHI database). Further, in the analysis of the TAP-independent peptide repertoire that is associated with non-classical MHC class I molecules, HLA-E- [39] and H-2Qa-1^b-bound peptides [42] were identified as length variants of the same central core. In the current report, multiple sets of up to ten nested peptides were endogenously presented by different HLA class I molecules (Table S4), suggesting that amino acid trimming at both the N- and C- terminus might be a general attribute of TAP-independent peptides. The similarities between the classical and non-classical peptide repertoires of HLA class II and TAP-independent HLA class I suggest at least a partial contribution of TAP-independent peptide loading in the post invariant chain-degrading compartments as indicated the existence of uncharacterized TAP-independent peptide-HLA-B27 complexes generated by a chloroquine-sensitive pathway [43].

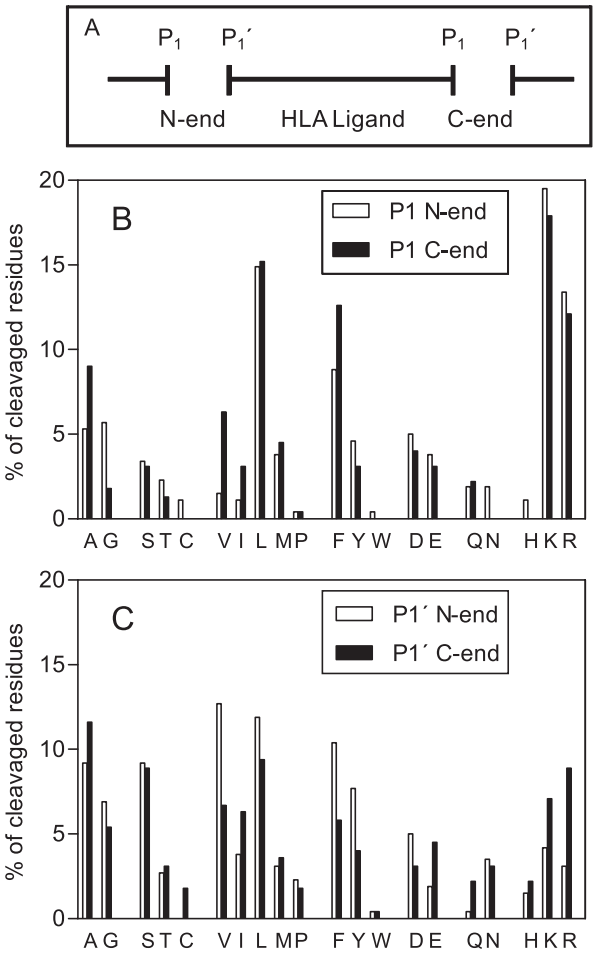

Figure 2. Analysis of N- and C-end cleavage specificity in HLA class I ligands. A diagram of residues involved in the generation of naturally processed HLA class I ligands by peptidase cleavages is shown (panel A). Distribution of P_1 (panel B) and P'_1 (panel C) amino acid residues of the scissile bonds created by peptidase cleavage.
doi:10.1371/journal.pone.0059118.g002

Unlike previous studies [8,9], the higher number of ligands identified in the current report allows the identification of extensive TAP-independent antigen processing mechanisms, yielding multiple HLA ligands in a reduced subgroup representing a small number of cellular proteins. Previously, autophagic processes present at low levels in different cell lines have been suggested to explain TAP-independent antigen processing [44]. In addition, as peptides derived from cytosolic proteins are found bound to class II MHC molecules [45–47], the mechanisms involved in peptides and/or protein transport from the cytosol to secretory pathways could be similar for both TAP-independent class I and class II MHC ligands. This possibility is supported by our identification of nested sets of TAP-independent peptides that are very similar to those previously reported to be associated with HLA class II molecules. The similar composition of the secretory vesicle proteome that includes both HLA class I and class II molecules [32], as well as the nested set TAP-independent ligands identified in the current report, suggest that the antigen processing of proteins from these or similar organelles could be a relevant source of the TAP-independent peptidome. This vision of MHC

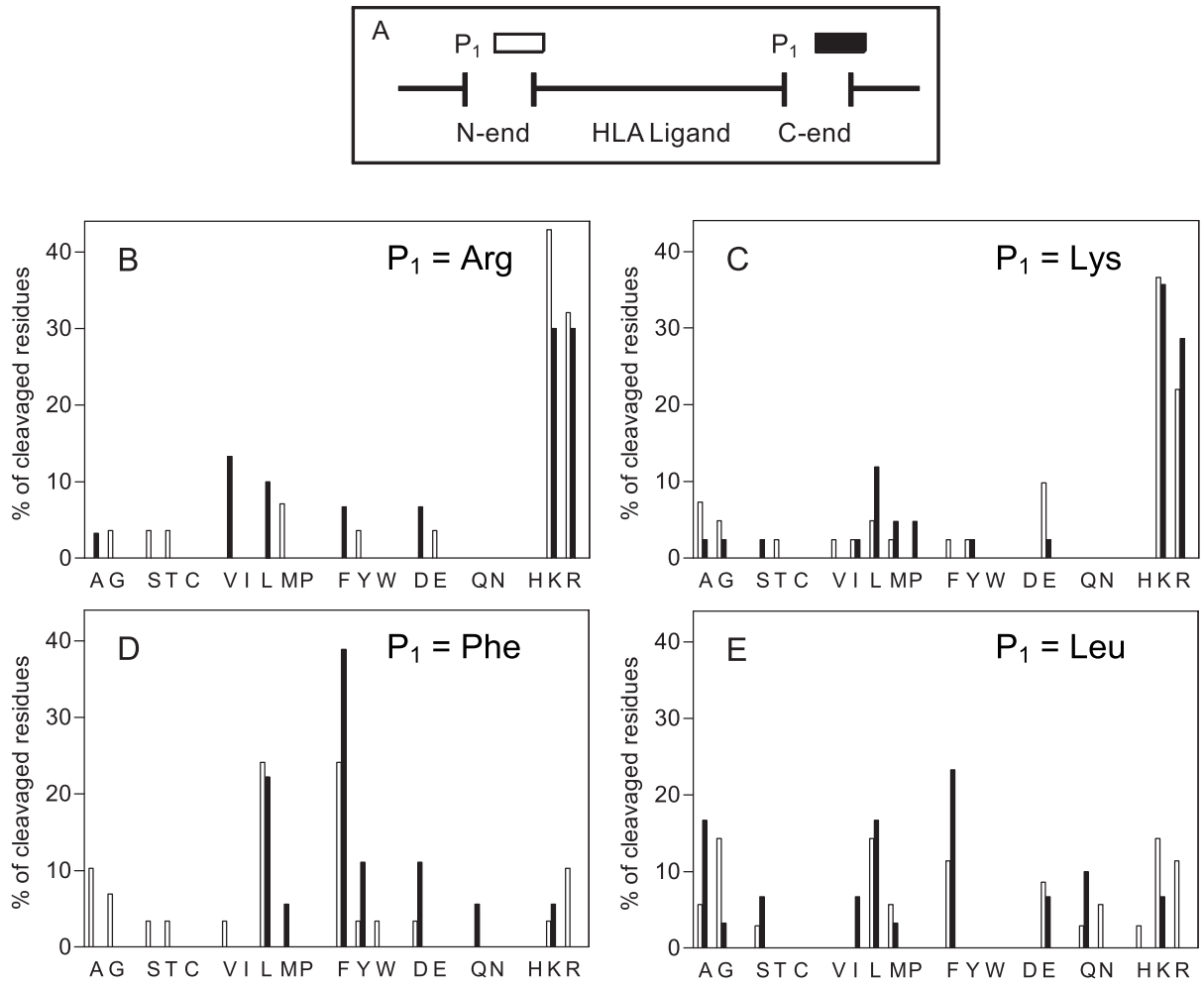

Figure 3. Analysis of the correspondence of P_1 N- and C-end cleavage specificity in HLA class I ligands. Panel A: A diagram of the residues involved in the generation of naturally processed HLA class I ligands by peptidase cleavage is shown. P_1 residue is indicated by white boxes (N-end) or black boxes (C-end). Panels B-E: A specific amino acid residue is indicated at the top right corner of each panel and the corresponding opposite residue identified in P_1 N-end (white boxes) or C-end (black boxes) is represented. For example, panel B indicates in white bars the residue located in the P_1 N-end position when Arg was identified in P_1 C-end, and in black bars the residue located in the P_1 C-end position when Arg was identified in the P_1 N-end position.
doi:10.1371/journal.pone.0059118.g003

class I molecules, from cell surface protein recycling, to entering into classical MHC class II compartments and later being transported back to the plasma membrane associated with endocytic/secretory vesicle peptides, might be the case for the peptides identified in the current report and is supported by two previous studies. At first, a measles virus F protein epitope was previously presented by class I molecules in TAP-independent, acidic-sensitive manner [48]. Second, uncharacterized TAP-independent peptide-HLA-B27 complexes were generated by a proteasome-independent, but chloroquine-sensitive, pathway [43]. Thus, this endocytic/secretory pathway may exist under normal conditions where it may contribute to a minor fraction of presented ligands. However, when the highly predominant TAP-dependent ligands are absent, as in TAP-deficient cells, this process may predominate.

Individual HLA class I alleles have shown different TAP dependencies. The prevalent HLA-A2 allele is considered to be the least TAP-dependent [49]. In contrast, other MHC class I molecules, including HLA-A3, -A24, and -B27, have been described as mainly TAP-dependent [26]. In the present report, a similarly broad TAP-independent peptidome was identified by MS for these HLA alleles with differing TAP requirements. Thus, quantitative rather than qualitative differences (probably associated with the high efficiency rate of the SPase, accounting for the majority of signal sequence cleavages [50]), are responsible for the diverse overall expression of various MHC class I molecules in TAP-deficient cells [43].

The global picture emerging from the current report is consistent with the model depicted in Figure 4. Some ligands, mostly HLA-A2-restricted, were processed by the SPase, in accordance with previous studies [9,43,51,52]. Endoproteolytic peptidases, exhibiting specificity for Arg/Lys or Phe/Leu in the P_1 position of the scissile bond, play an important role in the generation of many ligands associated with the four HLA class I alleles studied herein. However, some TAP-independent ligands must be produced by other, yet uncharacterized protease activities. Finally, a fraction of the longest variants from the same core protein could be folded within the HLA molecules, thereby protecting them from the trimming activity. The remaining molecules might be accessed by amino and/or carboxypeptidases

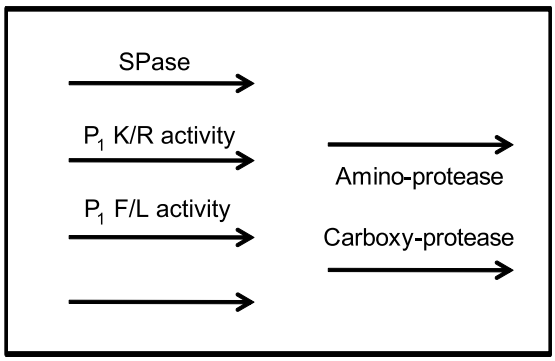

Figure 4. A model of the diversity of proteases and parallel processing pathways involved in TAP-independent self-derived antigen presentation. The model shows the components involved in each of the proposed pathways, with the relative order of the different steps. Involvement of SPase is deduced according to the SwissProt (http://web.expasy.org) and Signal P 4.1 (http://www.cbs.dtu.dk/services/SignalP) predictions. Involvement of P_1 K/R or F/L activities is deduced from Figures 2 and 3. The lower left arrow is deduced from unassigned ligands, and the amino and/or carboxyl peptidase activities could be assumed by analogy from previous studies.
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and trimmed to shorter peptides. Several rounds of trimming and HLA/peptide stabilization of fractions of these peptides could form the sets of nested peptides identified by mass spectrometry. Recurrent and sequential amino-terminal trimming and MHC protection have been demonstrated for a nested set of abundant and equally antigenic murine HLA class I epitopes from TAP-sufficient cells that ranged between 9–15 residues [53,54]. Whereas the role of the ER-resident peptidase ERAP in the N-terminal end trimming of different MHC class I ligands was previously well defined [55–57], to date, very few studies have implied a role for carboxypeptidases in antigen processing in the vesicular pathway. Indirect evidence has been reported in two cases. First, the proteolytic action of furin in the secretory pathway is required to generate an antigenic viral epitope [58]. After cleavage by furin, several C-terminal residues must be trimmed from the precursor peptide to generate the optimal epitope, suggesting that carboxypeptidases are involved. Second, several signal sequence-derived peptides generated by SPase complexes have C-terminal-extended residues when compared to the optimal HLA-bound epitope [8,9,42], indicating that carboxypeptidases may be involved in antigen processing of these ligands. A direct role for the carboxypeptidase ACE was described for the processing of peptides for MHC class I [59]. Recently, undefined carboxypeptidases were involved in the antigen processing of a vaccinia-derived TAP-independent epitope [60]. Finally, the present report indirectly implicates carboxyproteases in the generation of nested sets of peptides bound to several HLA class I alleles.

In summary, different and complex processing pathways are required to generate the HLA class I peptide repertoire in TAP-deficient cells.

Supporting Information

Figure S1 Diagram of sequential immunoprecipitation.
(PDF)

Figure S2 Identification of the $SF_{328–336}$ ligand in cell extracts by mass spectrometry.
(PDF)

Figure S3 Identification of the $LMMP5_{249–262}$ ligand in cell extracts by mass spectrometry.
(PDF)

Figure S4 HLA-B*2705 stabilization assay with synthetic ligands.
(PDF)

Figure S5 Length distribution of naturally processed peptides presented by HLA class I molecules in a TAP-deficient T2 cell line versus TAP-dependent ligands.
(PDF)

Figure S6 Representative nested set peptides of ligands identified by mass spectrometry.
(PDF)

Figure S7 Naturally processed peptides from MRCL2, β-actin, and glyceraldehyde 3-P dehydrogenase proteins identified by mass spectrometry.
(PDF)

Table S1 Summary of HLA-A2 ligands identified by mass spectrometry analysis.
(PDF)

Table S2 Summary of HLA-B27 ligands identified by mass spectrometry analysis.
(PDF)

Table S3 Summary of HLA-B51, or -Cw1 ligands identified by mass spectrometry analysis.
(PDF)

Table S4 Summary of HLA ligands clustered by protein origin.
(PDF)

Table S5 Subcellular location of proteins with TAP-independent ligands presented by HLA-A2, -B27, and -B51 or –Cw1.
(PDF)

Table S6 Summary of HLA ligands identified and coverage protein from individual protein.
(PDF)

Table S7 Summary of predominant cleavaged residues by peptidases in TAP-independent ligands.
(PDF)

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Author Contributions

Conceived and designed the experiments: DL. Performed the experiments: EL SI EB AB FL MJ. Analyzed the data: EL EB IB AA DL. Contributed reagents/materials/analysis tools: NG-M. Wrote the paper: DL.

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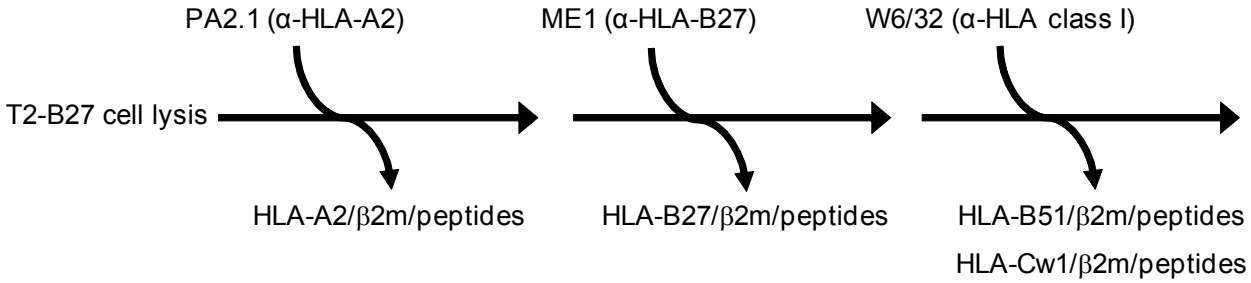
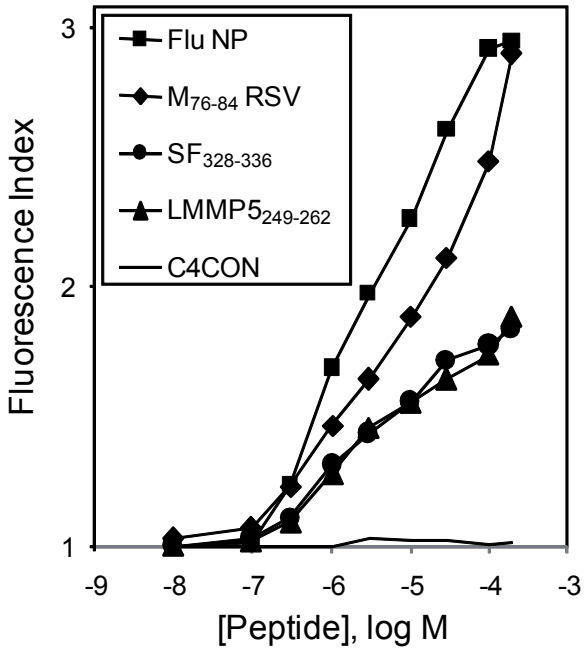


Lorente et al. Figure S1



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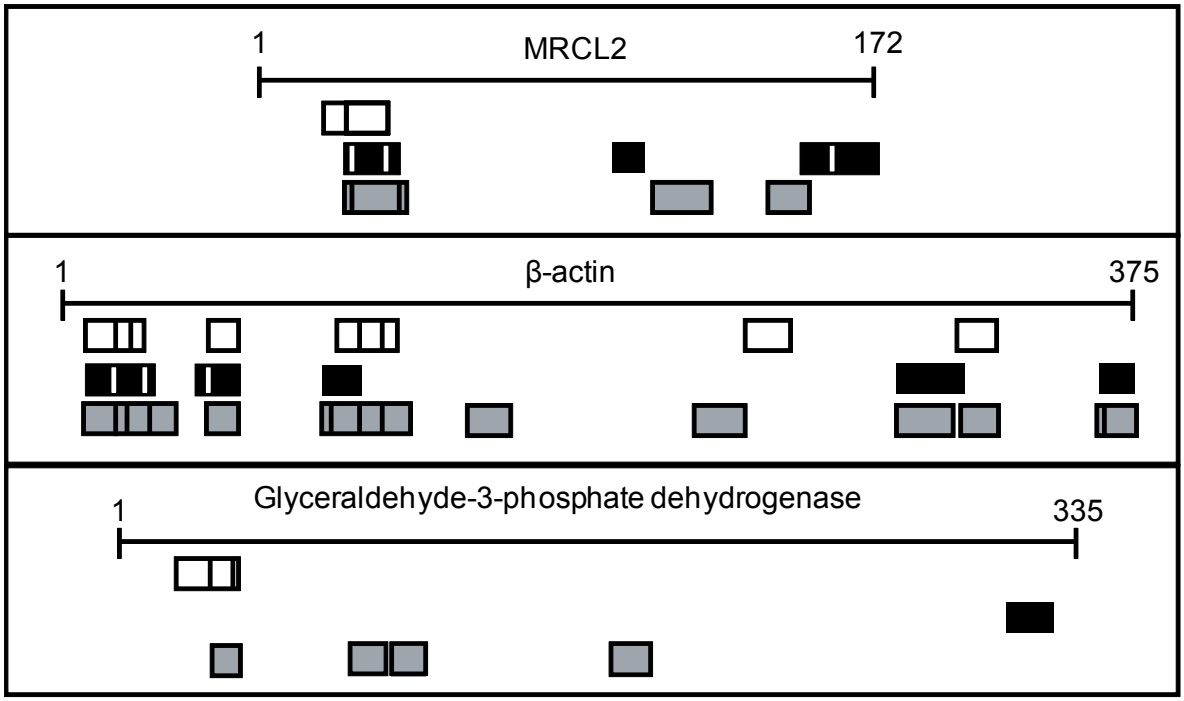

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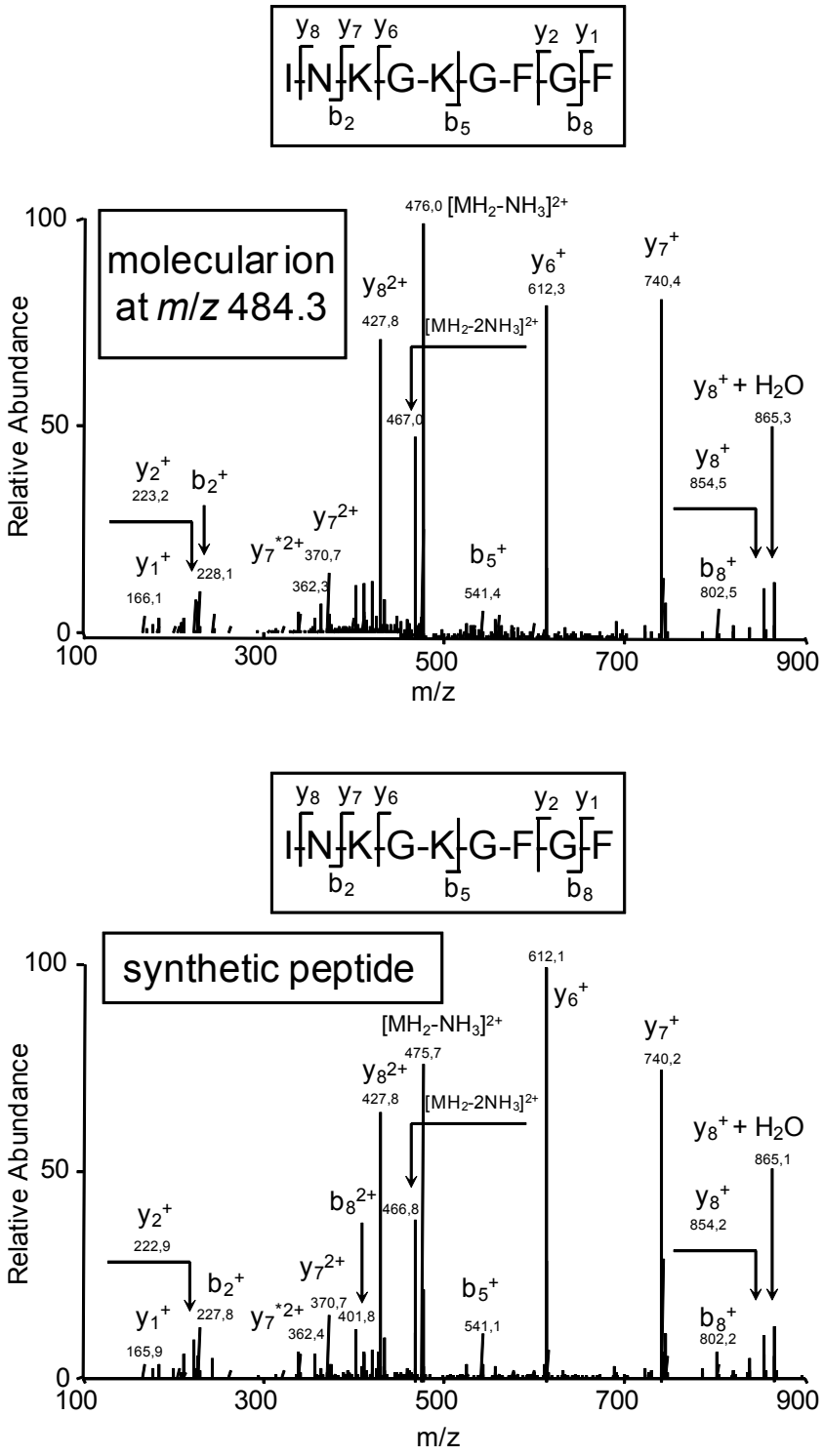

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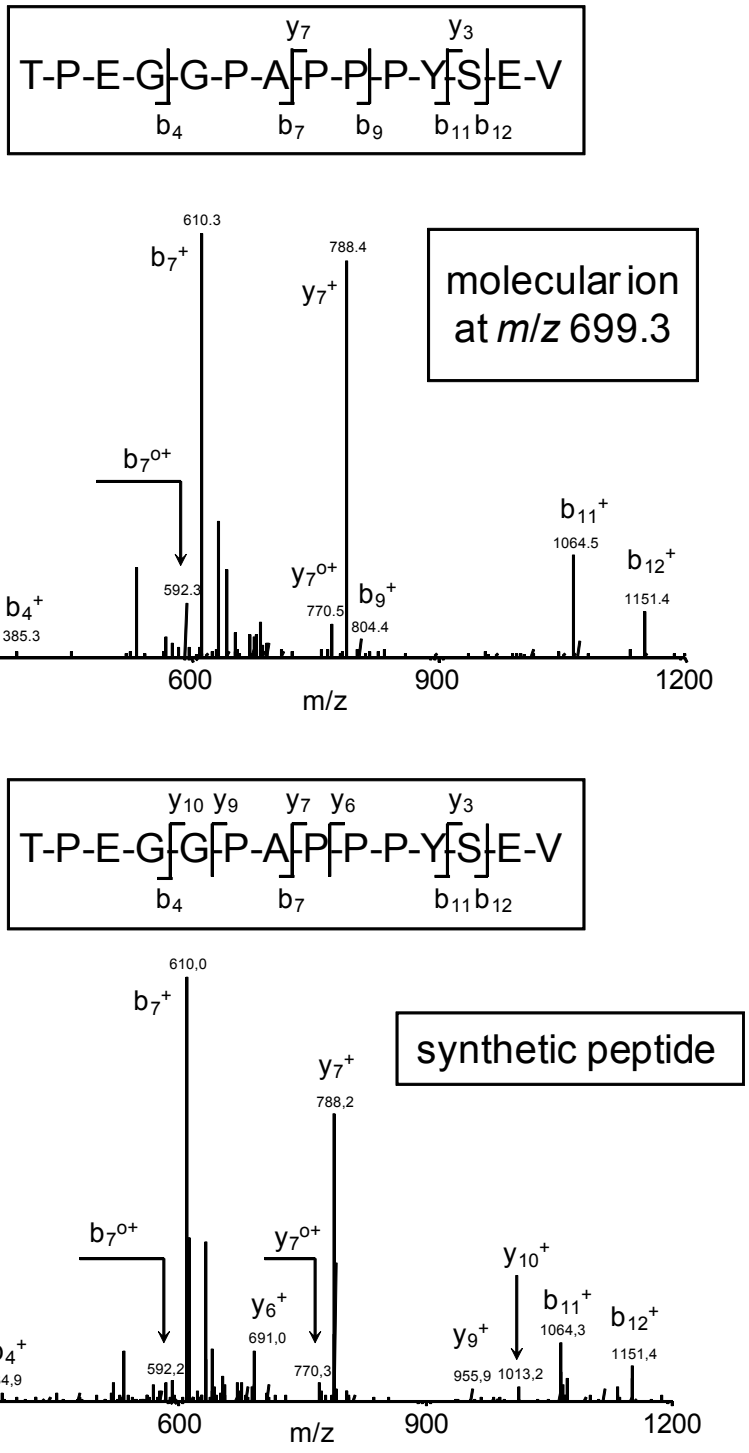

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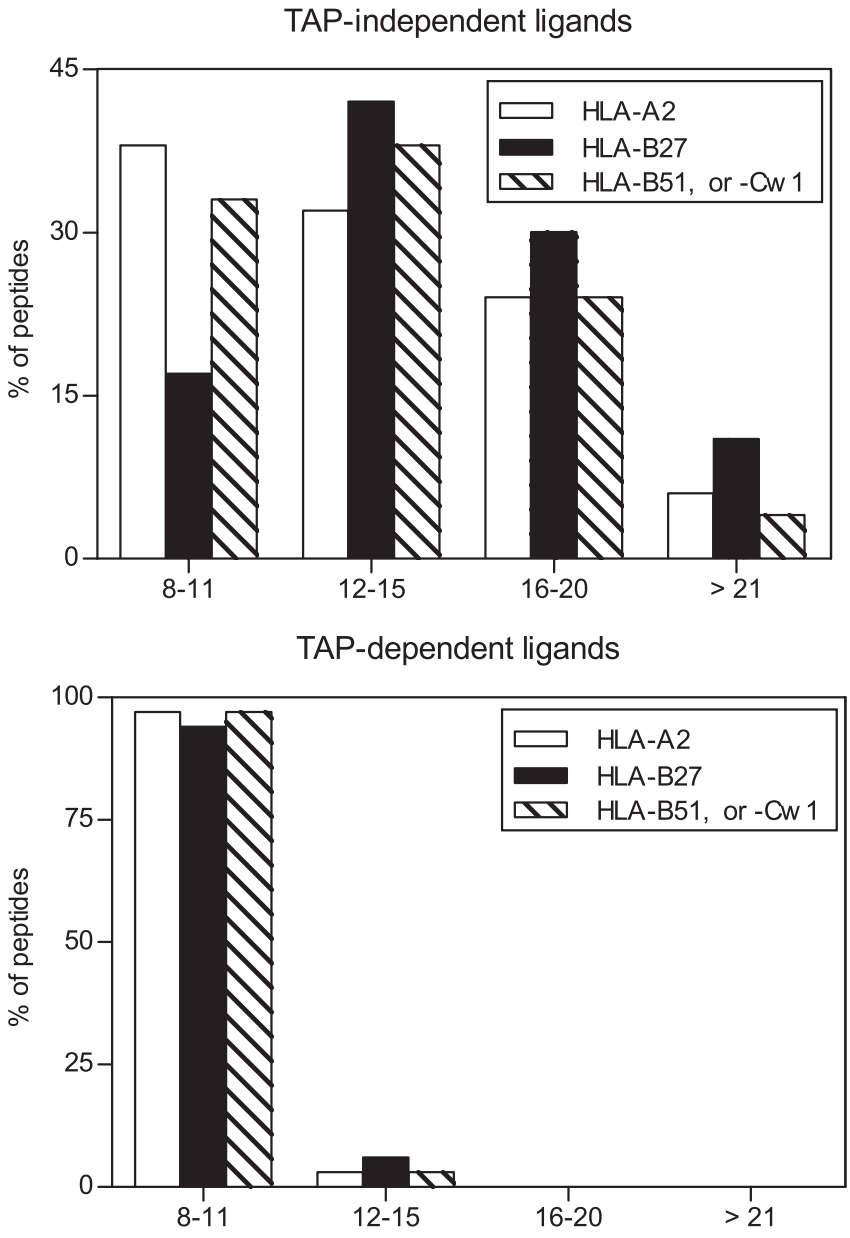

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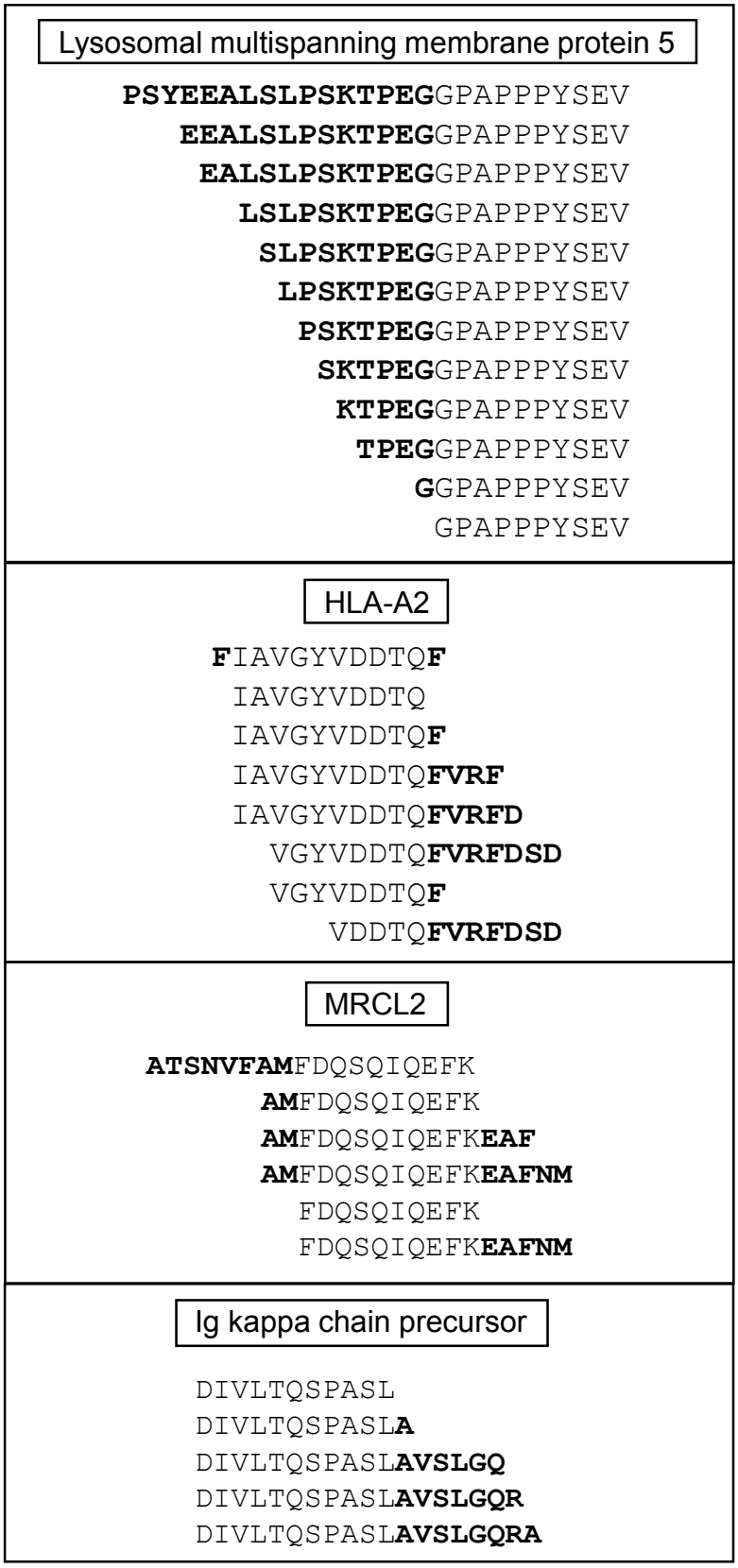

Lorente et al. Figure S6

Supplementary Table 5

Subcellular location of proteins with TAP-independent ligands presented by HLA-A2, -B27, and -B51 or –Cw1

Protein	Gi accession	Subcellular location [a]	N[er] of peptides
Beta Actin	4501885	cytoskeleton	24
CD74a	68448544	ER-Golgi-endosome [b]	7
Glyceraldehyde-3-phosphate dehydrogenase	7669492	cytosol	6
HLA-A2	717123	ER-Golgi-membrane	15
Heterogeneous nuclear ribonucleoprotein A1	133254	nucleus, cytoskeleton	5
Heterogeneous nuclear ribonucleoprotein U	126302554	nucleus, cytoskeleton	10
Myosin regulatory light chain MRCL2	15809016	cytoskeleton	11
Myosin, heavy polypeptide 9, non-muscle	12667788	cytoskeleton	30
TTD non-photosensitive 1 protein	20162566	nucleus	1
Tubulin alpha 6	14389309	cytoskeleton	3
Total peptides in these 10 proteins			112

[a] Cell location based on gene ontology analysis (http://www.geneontology.org)

[b] ER, endoplasmic reticulum.

Supplemental Table 7

Summary of predominant cleavaged residues by peptidases in TAP-independent ligands

Cleavaged residue	HLA-A2		HLA-B27		HLA-B51, -Cw1		Total HLA alleles	
	P_1 N-end [a]	P_1 C-end [a]	P_1 N-end [a]	P_1 C-end [a]	P_1 N-end [a]	P_1 C-end [a]	P_1 N-end [a]	P_1 C-end [a]
K/R	33 [b]	23	34	42	27	24	33	30
L/F	24	29	20	30	24	27	24	28
Σ	57	52	54	72	51	51	57	58

[a] from figures 6 and 7.

[b] Data are expressed as percentage of total cleavages.

SUPPORTING FIGURES LEGENDS

Figure S1. Diagram of sequential immunoprecipitation.
Healthy or VACV-infected T2-B27 transfectant cells (4 x 10^{10} cells) were lysed. HLA-peptide complexes were isolated via affinity chromatography from the soluble fraction of cell extracts with the following mAbs, used sequentially: PA2.1 (anti-HLA-A2), ME1 (anti-HLA-B27), and W6/32 (specific for a monomorphic HLA class I determinant).

Figure S2. Identification of the SF328-336 ligand in cell extracts by mass spectrometry.
MS/MS fragmentation spectra obtained from quadrupole ion trap mass spectrometry of the ion peak at m/z 484.3 from the cell extract (upper panel) and the corresponding synthetic peptide (lower panel). The vertical axis represents the relative abundance of the parental ion and each fragmentation ion detected. Ions generated in the fragmentation are detailed, and the sequence deduced from the indicated fragments is shown in the upper box of each panel.

Figure S3. Identification of the LMMP5249-262 ligand in cell extracts by mass spectrometry.
MS/MS fragmentation spectra obtained from quadrupole ion trap mass spectrometry of the ion peak at m/z 699.3 from the cell extract (upper panel) and the corresponding synthetic peptide (lower panel). The axis data are consistent with Supplemental Figure 2.

Figure S4. HLA-B*2705 stabilization assay with synthetic ligands.
The stability of HLA-B*2705/peptide complexes on the surface of RMA-S transfectant cells was measured by flow cytometry. The mAb used was the monoclonal ME1. The titration curves for synthetic SF328-336 (circles) and LMMP5249-262 (triangles) peptides with HLA- B*2705 are depicted. The C4CON (solid line) peptide was used as a negative control. The Flu NP (squares), and the RSV M76-84 (diamonds) peptides were used as positive controls. The results, calculated as the fluorescence index, represent the mean of four independent experiments.

Figure S5. Length distribution of naturally processed peptides presented by HLA class I molecules in a TAP-deficient T2 cell line versus TAP-dependent ligands.
Upper panel: Respective totals of 111, 77, and 192 TAP-independent ligands were co-immunoprecipitated with HLA-A2 (open bars), -B27 (filled bars), and –B51 or –Cw1 (dashed bars) class I molecules. Lower panel: Respective totals of 424, 571, 68, and 9 TAP-dependent ligands from HLA-A2 (open bars), -B27 (filled bars), and –B51 or –Cw1 (dashed bars) class I molecules (SYFPEITHI database and [28]).

Figure S6. Representative nested set peptides of ligands identified by mass spectrometry.
Diagram of representative nested set ligands N-extended (upper panel), C-extended (lower panel) or doubly extended (middle panels). The name of the protein source is boxed in each respective panel and the respective N- and C-extended residues are bolded.

Figure S7. Naturally processed peptides from MRCL2, β-actin, and glyceraldehyde 3-P dehydrogenase proteins identified by mass spectrometry.
Diagram of identified ligands bound to HLA class I molecules from MRCL2 (upper panel), β-actin (middle panel), and glyceraldehyde 3-P dehydrogenase (lower panel). Ligands specific for HLA-A2 (white boxes), -B27 (black boxes), and -B51 or -Cw1 (gray boxes) are depicted in each panel.

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Allele-dependent Processing Pathways Generate the Endogenous Human Leukocyte Antigen (HLA) Class I Peptide Repertoire in Transporters Associated with Antigen Processing (TAP)-deficient Cells*

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The transporters associated with antigen processing (TAP) allow the supply of peptides derived from the cytosol to translocate to the endoplasmic reticulum, where they complex with nascent human leukocyte antigen (HLA) class I molecules. However, infected and tumor cells with TAP molecules blocked or individuals with nonfunctional TAP complexes are able to present HLA class I ligands generated by TAP-independent processing pathways. These peptides are detected by the $CD8^+$ lymphocyte cellular response. Here, the generation of the overall peptide repertoire associated with four different HLA class I molecules in TAP-deficient cells was studied. Using different protease inhibitors, four different proteolytic specificities were identified. These data demonstrate the different allele-dependent complex processing pathways involved in the generation of the HLA class I peptide repertoire in TAP-deficient cells.

The newly synthesized proteome is sampled continuously by $CD8^+$ lymphocytes as short peptides presented by human leukocyte antigen (HLA) class I molecules at the cell surface. The majority of the peptides presented by HLA class I molecules are produced from proteolysis by the proteasome and other cytosolic proteases, such as tripeptidyl peptidase II (1–3), puromycin-sensitive aminopeptidase (4), insulin-degrading enzyme (5), thimet oligopeptidase (6), and caspases (7, 8). These peptides are transported into the endoplasmic reticulum (ER)[2] by TAP, with subsequent N-terminal trimming by the metalloaminoproteases ERAP1 and 2 frequently being required (9, 10). Peptide binding to nascent HLA class I molecules generates stable peptide-HLA complexes that are exported to the cell membrane where are they exposed to cytotoxic $CD8^+$ T lymphocyte recognition (for review, see Ref. 11).

$TAP^{-/-}$ humans (12) and mice (13) have a reduced functional $CD8^+$ population but do not appear to have an increased susceptibility to neoplasms or viral infections. Thus, the TAP-independent pathways may be sufficient to control these diseases and allow individuals with this HLA class I deficiency to live normal life spans with only a limited susceptibility to chronic respiratory bacterial infections. In addition, evidence for TAP-independent pathways of antigen presentation by MHC class I molecules of particular but diverse pathogenic epitopes was reported previously (for review, see Ref. 14–16). The identified proteases involved in the generation of specific ligands in TAP-deficient cells include ER signal peptidase (SPase) (17, 18), ER signal peptide peptidase (SPPase) (19, 20), trans-Golgi network furin (21, 22), and lysosomal cathepsins (23). However, systematic studies of TAP-independent pathways involved in the generation of the overall peptide repertoire associated with different HLA class I molecules have not been reported. Studying the reexpression of newly synthesized complexes of different HLA class I molecules in the presence of diverse protease inhibitors allowed the determination of several allele-dependent processing pathways in TAP-deficient cells.

EXPERIMENTAL PROCEDURES

Cell Lines—T2 is a TAP-deficient human cell line that express HLA-A2, -B51, and -Cw1 class I molecules on the cell surface (24). T2-B27 cells were generated by transfection of T2 cells with HLA-B27 (a gift from Dr. David Yu, University of California, Los Angeles). T2 and T2-B27 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum.

Chemicals—Brefeldin A (BFA), chloroquine (CQ), and all protease inhibitors were from Sigma-Aldrich except leupeptin (Amersham Biosciences), pepstatin (Roche Applied Science), butabindide (Tocris), decanoyl-Arg-Val-Lys-Arg-CMK (dec-RVKR) (Bachem), $(z\text{-}LL)_2$ ketone (Merck), and lactacystin (Dr. E. J. Corey, Harvard University). The general specificity and activity of all inhibitors used in this study are summarized in Table 1. The normal HLA class I re-expression of at least one allele for each inhibitor demonstrates no toxic effect by the use of different inhibitors (see below).

Acid Stripping and HLA Class I Reexpression—Cells were washed with RPMI 1640 medium in the absence of serum and incubated for 90 s with ice-cool acid-stripping medium (0.3 M glycine HCl and 1% BSA in water, pH 2.4) as reported previously (25). Culture medium was added to neutralize the pH. Cells were washed three times, resuspended in assay medium (RPMI 1640 medium with 1% BSA) at 10^6/ml, and incubated at 37 °C for 6 h in the presence or absence of inhibitors at the

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[2] The abbreviations used are: ER, endoplasmic reticulum; BFA, brefeldin A; CQ, chloroquine; SPase, ER signal peptidase; SPPase, ER signal peptide peptidase; TAP, transporters associated with antigen processing.

TABLE 1
General specificity of inhibitors used in this study

Inhibitor	Abbreviation	Specificity	Reference	Concentration
Brefeldin A	BFA	Vesicle transport	30, 31	5 μg/ml
Lactacystin	LC	Proteasome chymotryptic and tryptic activities	32–34	10 μM
Epoxomicin	EPOX	Proteasome chymotrypsin-like activity	35, 36	1 μM
E64	E64	Cysteine proteases C1	39	100 μM
Leupeptin	LEU	Trypsin-like proteases and cysteine proteases	37	100 μM
Pepstatin	PEPST	Aspartic proteases	37, 38	100 μM
1,10-Phenanthroline	PHE	Metalloproteases and caspase-1	38, 40	50 μM
Leucinthiol	LeuSH	Metallo-aminopeptidases including ERAAP	43	30 μM
Butabindide	BUT	Tripeptidyl peptidase II	56	100 μM
Puromycin	PUR	Dipeptidyl-peptidase II and puromycin-sensitive aminopeptidase	57	0.5 μg/ml
Decanoyl-Arg-Val-Lys-Arg-CMK	dec-RVKR	Furin and other members of the SPC family	58	100 μM
$(z\text{-}LL)_2$ ketone	$z\text{-}LL_2$	Signal peptide peptidase	19, 20	100 μM
Chloroquine	CQ	Lysosomotropic agent	47, 48	50 μM

indicated concentrations (Table 1). Serum-free conditions were used throughout.

Flow Cytometry—HLA expression levels were measured using the monoclonal antibodies (Abs) ME1 (anti-HLA-B27) (26) in T2-B27 cells; polyclonal H00003106-B01P (specific for HLA-B class I molecules; Abnova) in T2 cells; and monoclonal PA2.1 (anti-HLA-A2) (27) and polyclonal SC-19438 (specific for HLA-C class I molecules; Santa Cruz Biotechnology) in T2 and T2-B27 cells simultaneously as described previously (28). Samples (10^4 cells) were run on a FACSCanto flow cytometer (BD Biosciences) and analyzed using CellQuest Pro 2.0 software (BD Biosciences). Percentage of inhibition of HLA reexpression obtained by the addition of the indicated inhibitors (Table 1) was calculated utilizing the following equation.

$$\%\ \text{Inhibition} = 100 - \frac{\text{MFI}_{+\text{inhibitor}} - \text{MFI}_{+\text{2nd Ab}}}{\text{MFI}_{\text{without inhibitor}} - \text{MFI}_{+\text{2nd Ab}}} \times 100 \quad \text{(Eq. 1)}$$

Statistical Analysis—To analyze statistical significance, an unpaired Student's *t* test was used. p values < 0.01 were considered significant.

RESULTS

Endogenous Processing of TAP-independent HLA Ligands—To examine the generation of the TAP-independent HLA-bound peptide repertoire, TAP-deficient T2 and T2-B27 cells, treated with acid to remove surface class I peptide complexes, were allowed to reexpress newly synthesized complexes for 6 h. The T2-B27 cell line was selected because it expresses one each of the endogenous HLA-A, -B, and -C alleles and an additional HLA-B class I molecule, thus mimicking a partial heterozygous haplotype. This expression pattern allows four different class I molecules to be studied. As reported previously for some MHC class I alleles (29), different reexpression levels were found in each of the four HLA class I molecules studied (Fig. 1, *upper*). To test whether TAP-independent ligands require endogenous processing, HLA reexpression was analyzed in the presence of BFA. This drug blocks class I export beyond the cis-Golgi compartment (30, 31), preventing the surface expression of newly assembled class I-peptide complexes from endogenous origin. The reexpression level of all four HLA class I alleles expressed by the TAP-deficient cells used was decreased significantly: 84% ± 10% for HLA-A2, 87% ± 8% for HLA-B27, 80% ± 7% for HLA-B51, and 63% ± 8% for HLA-Cw1 (Fig. 1). These results

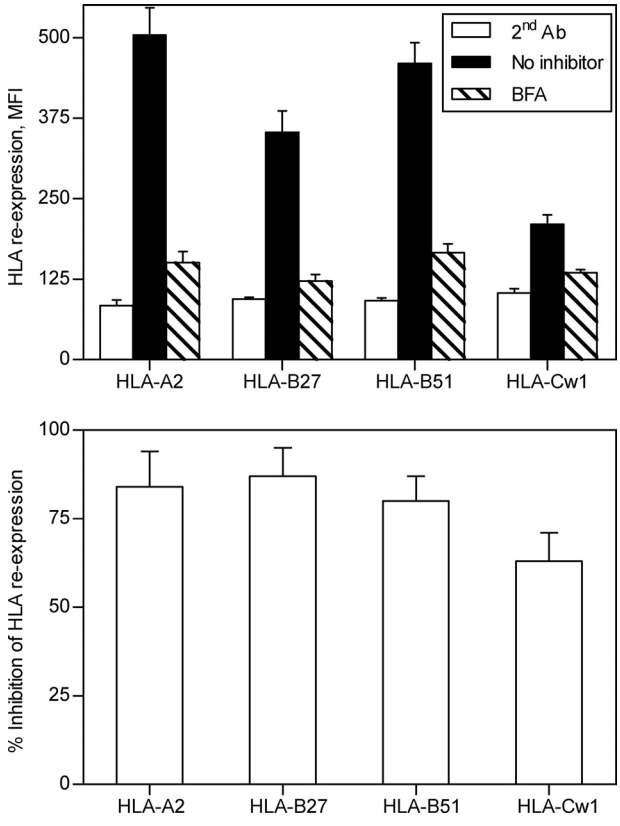

FIGURE 1. **Surface reexpression of HLA class I molecules after acid stripping in the presence of BFA.** T2 and T2-B27 cells were untreated (*black bars*) or incubated with 5 μg/ml BFA for 6 h (*hatched bars*) after acid washing. Stability at the cell surface of HLA-A2, -B27, -B51, and -Cw1 class I molecules of the TAP-deficient cells was measured by flow cytometry using monoclonal Ab ME1 (anti-HLA-B27) in T2-B27 cells; polyclonal H00003106-B01P (specific for HLA-B class I molecules) in T2 cells; and monoclonal Abs PA2.1 (anti-HLA-A2) and polyclonal SC-19438 (specific for HLA-C class I molecules) in T2 and T2-B27 cells simultaneously. The data are expressed as MFI ± S.D. (*error bars*) (*upper*) or percentage of inhibition ± S.D. (*lower*) of HLA surface reexpression in presence of BFA and are the means of three or four different experiments.

demonstrated that the generation of the TAP-independent HLA-ligands was generated primarily from proteins endogenously processed in TAP-deficient cells.

Proteasome Inhibitors Differentially Affect the TAP-independent Expression of Distinct HLA Class I Molecules—To study the involvement of different proteolytic activities in the generation of TAP-independent ligands presented by HLA class I molecules, the surface reexpression of different HLA class I molecules after acid stripping in T2-B27 TAP-deficient cells

TABLE 2
Summary of inhibition of HLA reexpression

Alelle	BFA[a]	LC/EPOX	LeuSH	z-LL_2	CQ
HLA-A2	+[b]	+	+	+	−
HLA-B27	+	−	+	−	+
HLA-B51	+	+	+	+	−
HLA-Cw1	+	−	−	−	−

[a] For specificity of different inhibitors see Table I.
[b] + and − indicate percentage inhibition >35% and <10%, respectively. All + inhibitions show significant p values ($p < 0.01$) *versus* controls without an inhibitor.

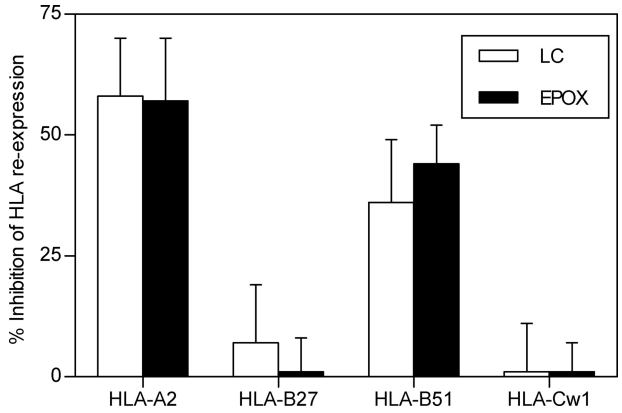

FIGURE 2. **Effect of several proteasome inhibitors on surface reexpression of HLA class I molecules after acid washing.** T2 and T2-B27 cells as in Fig. 1 were incubated with lactacystin (*open bars*) or epoxomicin (*filled bars*) at the indicated concentrations (Table 1) as in Fig. 1. The data are expressed as percentage of inhibition ± S.D. (*error bars*) as in Fig. 1 and are the means of four or five different experiments.

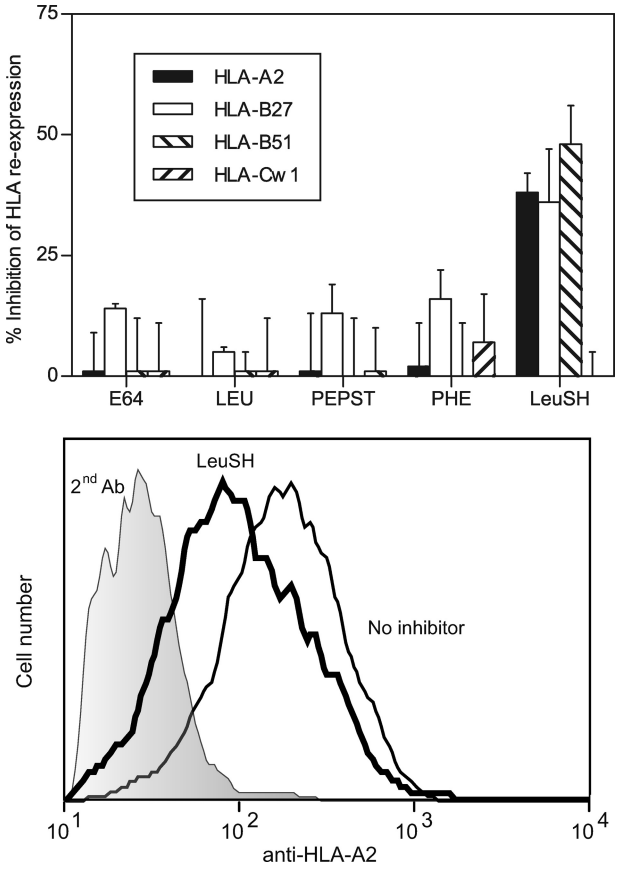

FIGURE 3. **Surface reexpression of HLA class I molecules after acid stripping in the presence of several protease classes inhibitors.** *Upper*, T2 and T2-B27 cells as in Fig. 1 were incubated with the indicated inhibitors at the concentrations summarized in Table 1. HLA-A2 (*filled bars*), -B27 (*open bars*), -B51 (*right hatched bars*), and -Cw1 (*left hatched bars*) surface reexpression was measured. The data are expressed as percentage of inhibition ± S.D. (*error bars*) as in Fig. 1 and are the means of three to six different experiments. *Lower*, a representative experiment with T2 cells stained with anti-HLA-A2 Ab is depicted. The code used is as follows: *shaded histogram*, second Ab alone (negative control); *thin line*, no inhibitor; and *thick line*, 30 μM leucinethiol.

was performed in the presence or absence of different inhibitors. Previously, partial block (~50% of inhibition) of HLA-A2 reexpression in TAP-deficient cells caused by the addition of lactacystin, a *Streptomyces* metabolite (32–34) (Table 2), demonstrated a role for proteasomes in HLA class I processing of TAP-independent HLA-A2 ligands (25). The involvement of multicatalytic complex proteasome in the processing of ligands presented by other HLA class I was studied. Both lactacystin and epoxomicin (35, 36), another proteasome inhibitor, partially block both HLA-A2 and -B51 reexpression (Fig. 2), implicating the proteasomes in the generation of HLA ligands presented by these alleles By contrast, in the same experiment, both proteasome inhibitors have no effect on the reexpression of HLA-B27 and -Cw1 class I molecules. Thus, these data indicate that the proteasome activity is not absolutely required to generate ligands bound to HLA-B27 and -Cw1 class I molecules.

Metallo-aminopeptidase Inhibitors Specifically Block the TAP-independent Expression of Peptide-HLA-A and -B but Not -C Complexes—To characterize proteases distinct from proteasomes that may contribute to processing of HLA ligands, experiments with several specific protease inhibitors were performed. Leupeptin (37), pepstatin (37, 38), E64 (39), and 1,10-phenanthroline (38, 40) inhibitors were initially tested because they are specific for different protease families (Table 1) and cover a wide range of protease classes. These four inhibitors had no effect on the HLA reexpression of any of the four alleles studied (Fig. 3). Thus, the enzymes inhibited by these drugs cannot be formally involved in the generation of TAP-independent ligands.

In addition, as the activity of ERAP, an enzyme previously involved in antigen processing (41, 42), is not fully blocked by 1,10-phenanthroline at the concentration used in this study, the inhibitor leucinethiol (Table 2) (43) was used. A partial inhibition of surface reexpression of HLA-A2 (38% ± 4%), -B27 (36% ± 10%), and -B51 (48% ± 8%) but not HLA-Cw1 (0% ± 5%) in TAP-deficient cells treated with leucinethiol was found (Fig. 3). These inhibitions are similar to those reported previously in TAP-sufficient cells, where the surface quantities of the murine MHC class I molecules, K^k and L^d, decreased between 20 and 40% by specific ERAP inhibition (43). Thus, these data implicate ERAP, or other metallo-aminopeptidases, in the generation of a subset of TAP-independent ligands presented by three of four HLA class I molecules examined.

SPPase Is Involved in the Generation of TAP-independent HLA-A2 and -B51 Ligands—Different proteases such as tripeptidyl peptidase II (1, 2), puromycin-sensitive aminopeptidase (4), and furin (21, 22) were previously implicated in antigen processing as being able to generate pathogen-derived peptides. The possible role of these enzymes in endogenous presentation of TAP-independent ligands was studied using available specific inhibitors (Table 1). Inhibition of HLA reex-

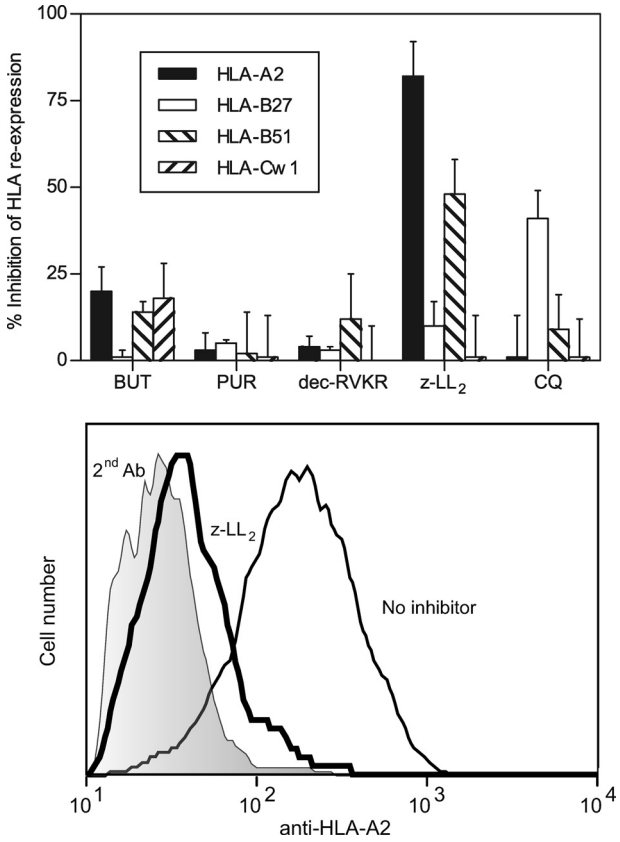

FIGURE 4. **Outcome of different protease-specific inhibitors or a lysosomotropic agent on surface reexpression of HLA class I molecules after acid washing.** *Upper*, T2 and T2-B27 cells as in Fig. 1 were incubated with the indicated drugs at the concentrations summarized in Table 1. HLA-A2 (*filled bars*), -B27 (*open bars*), -B51 (*right hatched bars*), and -Cw1 (*left hatched bars*) surface reexpression was calculated. The data are expressed as percentage of inhibition ± S.D. (*error bars*) as in Fig. 1 and are the means of three to six different experiments. *Lower*, a representative experiment with T2 cells stained with anti-HLA-A2 Ab is depicted. The code used as in follows: *shaded histogram*, second Ab alone; *thin line*, no inhibitor; and *thick line*, 100 μM z-LL_2.

pression was not detected with any of these drugs (Fig. 4) in all HLA class I alleles studied, and thus, the enzymatic activity of these peptidases is not absolutely required in the generation of TAP-independent ligands.

Different endogenous TAP-independent HLA-A2 class I ligands are derived by cleavage of the respective signal sequences generated by the SPase complex (16, 44). A specific inhibitor of this enzymatic activity is unavailable; thus, direct involvement of SPase complex in the generation of TAP-independent HLA-A2 ligands could not be studied. However, two SPase-processed peptides need further cleavage by SPPase (19, 20), allowing the hypothetical involvement of SPPase to be examined by treating TAP-deficient cells with the SPPase-specific inhibitor $(z\text{-}LL)_2$ ketone (19, 20). This drug specifically inhibits TAP-independent HLA reexpression of HLA-A2 (82% ± 10%), and -B51 (48% ± 10%), but not -B27 (10% ± 7%) or -Cw1 (1% ± 12%). These results demonstrate the role of SPPase in the generation of TAP-independent ligands for some HLA class I molecules.

TAP-independent Expression of Peptide-HLA-B27 Complexes Is CQ-sensitive—Previously, three epitopes processed in an endosomal/lysosomal antigen processing pathway for murine MHC class I presentation in TAP-deficient cells were blocked in cells treated with CQ (23, 45, 46). Thus, the contribution of the CQ-sensitive (47, 48) processing pathway to the generation of overall TAP-independent ligands was evaluated. In our experiments, specific reduction (41% ± 8%) of the HLA-B27 surface levels in presence of CQ was detected (Fig. 4). In contrast, the reexpression of HLA-A2, -B51, and -Cw1 class I molecules was not altered by this drug (Fig. 4). These data indicate that the endosomal/lysosomal antigen processing pathway is HLA class I allele-dependent.

Summary of Inhibitions of HLA Class I Reexpression—With the drugs used in this study (Table 1), three different inhibition patterns of HLA class I expression were found (summarized in Table 2). The inhibition obtained in all HLA-class I alleles examined in presence of BFA indicates that most of the TAP-independent HLA-bound peptides were endogenously processed. The surface levels of HLA-A2 and -B51 class I were dependent on proteasome, metallo-aminopeptidases (probably ERAP), and SPPase activities. Metallo-aminopeptidases and CQ-sensitive processing are relevant for generating HLA-B27 ligands. By contrast, none of the compounds used in this study decreased HLA-Cw1 surface expression.

DISCUSSION

This study was undertaken to compare the generation of the peptide repertoire associated with four different HLA class I molecules in TAP-deficient cells. Previously, the expression of various HLA class I molecules was differentially affected by proteasome inhibitors in TAP-sufficient cells (25). These inhibitors blocked the reexpression of HLA-A2 (60%) and -B51 (80%) class I molecules. In contrast, HLA-B27 was largely insensitive to proteasome inhibitors (only 30% of inhibition of reexpression) (25). In the same study, the role of the proteasome in processing TAP-independent HLA-A2 ligands was reported (25). We found that a different MHC class I molecule, HLA-B51, is also proteasome-dependent in the generation of the HLA peptide repertoire in TAP-deficient cells. In addition, the proteasome inhibitors have no effect on the expression of TAP-independent peptide/HLA-B27 or -Cw1 repertoires. Thus, differential involvement of the proteasome in the generation of ligands bound to HLA class I molecules was found in this study. There was a direct correlation of the role of proteasomes in the processing of HLA peptide repertoires of TAP-dependent and -independent ligands between TAP-sufficient and -deficient cells.

The role of SPase in the processing of TAP-independent presented HLA-A2 peptides has been found previously (17, 18, 44). Moreover, SPPase catalyzes intramembrane proteolysis of two signal peptides after they have been cleaved from a preprotein as the signal sequence-derived HLA-E peptides (19, 20). Thus, sequential cleavage by SPase and SPPase was involved in the processing for some peptides. We found that the specific inhibitor for SPPase activity significantly decreased the HLA expression of some alleles in TAP-deficient cells involving these sequential enzymatic activities in the processing of TAP-independent ligands bound to HLA-A2 and -B51 class I molecules.

It is well documented that trimming of ligand precursors in the ER is important for the generation of appropriate peptides

for HLA class I binding and that ER-resident aminopeptidase activity has an important impact on the repertoire of ligands presented in TAP-sufficient cells (for review, see Ref. 49). The present study demonstrates that metallo-aminoprotease-sensitive trimming is also required to generate TAP-independent ligands presented by different HLA class I molecules.

HLA-A2 and -B51 class I molecules demonstrated a similar HLA expression inhibition pattern involving the proteasome, SPPase, and metallo-aminopeptidase enzymatic activities in the generation of TAP-independent ligands. Thus, the most likely explanation is summarized via the following model. Multiple endogenous proteins are proteolyzed by the proteasome in the cytosol. Some of the generated peptides are released into the ER as indicated by the presentation in some instances of cytosolic proteins in cells lacking TAP (50, 51). This presentation of peptides could occur by passive diffusion (52), hydrophobic peptides with specific ability to traverse membranes (53), or unidentified transport. In parallel, SPase releases peptides with signal sequence into the ER. Finally, a fraction of total ER-peptides could be processed by SPPase activity and/or ERAP trimming prior to their binding to HLA-A2 or -B51 molecules.

The presentation pathway for extracellular antigens requires endocytosis and degradation in endolysosomal compartments. HLA class II molecules bind the peptides generated, and these complexes are transported to the cell surface (54). Reagents that prevent endosomal acidification block this type of processing, inhibiting the protein cleavage by endosomal enzymes, such as the acidophilic amine CQ. Previously CQ-sensitive endosomal processing of internalized endogenous transmembrane proteins (23) or viral particles (45) for the generation of murine MHC class I-binding peptides was reported. The current study shows the formation of the overall TAP-independent peptide-HLA-B27 complexes by a proteasome-independent but BFA- and CQ-sensitive pathway. These data imply that class I molecules on the cell surface are internalized to an endolysosomal compartment where they intersect with peptides either supplied by the lysosomal polypeptide transporter ABCB9, also named TAP-like (TAPL) (for review, see Ref. 55) or those directly processed by lysosomal proteases. In addition, some of these HLA-B27-restricted peptides need further trimming by metallo-aminopeptidases sensitive to leucinethiol.

In addition, inhibition of the HLA-Cw1 surface expression was not detected with the chemicals used in this study. Thus, the identification of the different peptidase(s) of the antigen processing pathway involved in the generation of HLA-Cw1 ligand awaits further molecular and cellular biology studies.

In summary, different and complex processing pathways involving at least four diverse proteolytic specificities in miscellaneous subcellular locations are required to generate the HLA class I peptide repertoire in TAP-deficient cells.

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Research Letters

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TAP-independent human histocompatibility complex-Cw1 antigen processing of an HIV envelope protein conserved peptide

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Individuals with nonfunctional transporters associated with antigen processing (TAP) complexes are not particularly susceptible to viral infections or neoplasms. Therefore, their immune system must be reasonably efficient, and the present, though reduced, cytolytic $CD8^+$ αβ T subpopulation specific for TAP-independent antigens may be sufficient to establish an immune defense protecting against viral infections in these individuals. The objective of the present study was to identify TAP-independent ligands from HIV gp160 protein. An analysis and comparison of complex human histocompatibility complex (HLA)-bound peptide pools isolated from large quantities of healthy or HIV gp160-expressing human cells was performed using mass spectrometry and bioinformatics tools. A conserved TAP-independent HLA peptide ligand endogenously processed and presented in infected human cells was identified. This ligand originates from the envelope protein bound to the HLA-Cw1 class I molecule with high affinity. It was concluded that HLA class I peptides derived from a large fraction of the N-terminal HIV envelope protein could be presented even in the absence of the TAP complex.

The killing of infected cells by $CD8^+$ cytolytic T lymphocytes requires previous proteolytic degradation of viral proteins [1]. This antigen processing generates short peptides that are translocated to the endoplasmic reticulum lumen by transporters associated with antigen processing (TAP), where they assemble with newly synthesized β_2-microglobulin and human histocompatibility complex (HLA) class I heavy chain. Following the initial assumption that the multicatalytic and ubiquitous proteasome is the only protease proficient in fully generating peptide ligands for HLA class I molecule binding, several studies have identified a growing number of alternative pathways that also contribute to endogenous antigen processing (reviewed in [2,3]). Individuals with mutations in the *TAP* gene that generate nonfunctional TAP complexes have been described (reviewed in [4]). Individuals with this HLA class I deficiency may be asymptomatic for long periods. Because TAP-deficient patients are not particularly susceptible to viral infections or neoplasms, their immune systems must be reasonably efficient. These individuals have sufficient repertoires of antibodies, natural killer (NK) cells, and $CD8^+$ γδ T cells, but a reduced cytolytic $CD8^+$ αβ T subpopulation specific for TAP-independent antigens, which together contribute to an immune defense that protects against severe viral infections. In two classic studies, Siliciano and colleagues [5,6] identified two nested TAP-independent epitopes in the HIV gp160 protein: residues 31–40 and 37–46 restricted by HLA-B18 and HLA-A3 class I molecules, respectively. No subsequent studies have addressed the existence of new TAP-independent ligands in this protein. To expand on the work by Siliciano and colleagues, we conducted a comparative immunoproteomic analysis of HLA ligands isolated from large quantities of TAP-deficient untreated or HIV gp160-expressing human cells. In this report, we describe the identification of yet another TAP-independent, HLA-Cw1-restricted, naturally processed ligand from the HIV gp160 protein.

HLA-bound peptides were isolated from 4×10^{10} healthy or recombinant vaccinia virus vSC25-infected T2-B27 transfectant cells as previously described [7]. T2, a line of TAP-deficient human cells that express HLA-A2, HLA-B51, HLA-Cw1, and HLA-E class I molecules on their surface [8], was transfected with HLA-B27 (a gift from Dr David Yu, University of California, Los Angeles, California, USA). The vaccinia vector vSC25 encodes the envelope (ENV) glycoprotein gp160 from the HIV-1 strain IIIB [9] inserted in the genome of the western reserve strain. HLA-peptide complexes were isolated via affinity chromatography of the soluble fraction of cell extracts with the following monoclonal antibodies (mAbs) used sequentially: PA2.1 (anti-HLA-A2) [10], ME1 (anti-HLA-B27) [11], and W6/32 (specific for a monomorphic HLA class I determinant) [12].

HLA class I peptides immunoprecipitated with each HLA-specific mAb were analyzed in three high-pressure liquid chromatography (HPLC) runs by micro liquid chromatography-mass spectrometry (μLC-MS/MS) using an Orbitrap XL mass spectrometer (Thermo-Fisher, San Jose, California, USA) [7]. Bioworks Browser 3.3.1 SP1 (Thermo-Fisher) was used for peak-list generation of the μLC-MS/MS data, and the HLA peptides were identified using the Sequest software tool and the human and virus parts of the NCBI database (January 2009), which includes 656 486 proteins. Identified peptides were selected if the following criteria were met: Sequest Xcorr more than 1.4 for singly, more

than 2.2 for doubly, and more than 2.9 for triply charged peptides; P(pep) less than 1×10^{-3}, and mass accuracy of 0.005 Da [7]. The purpose of the filtering criteria was to identify candidate HIV gp160 peptide MS/MS scans for further manual inspection to determine whether the MS/MS fragment ion fingerprint matched the identified peptide sequence. In addition, the corresponding synthetic peptide was made, and its MS/MS spectrum was used to confirm the assigned sequence.

The following synthetic peptides were used as controls in HLA/peptide complex stability assays: KPNA2 (GLVPFLVSV, HLA-A2-restricted) [13], HBV HBc_{19-27} (LPSDFFPSV, HLA-B51-restricted) [14], CMV $pp65_{7-15}$ (RCPEMISVL, HLA-Cw1-restricted) [15], HLA-A2 peptide leader (VMAPRTLVL, HLA-E-restricted), and C4CON (QYDDAVYLK, HLA-Cw4-restricted) [16]. The T2 line of TAP-deficient cells was used as previously described [17]. HLA expression levels were measured using the Abs monoclonal PA2.1 (anti-HLA-A2), monoclonal 3D12 (anti-HLA-E) [18], polyclonal H00003106-B01P (specific for HLA-B class I molecules; Abnova, Taipei, Taiwan), and polyclonal SC-19438 (specific for HLA-C class I molecules; Santa Cruz Biotechnology, Santa Cruz, California, USA) as previously described [17]. The fluorescence index was calculated as the ratio of the mean channel fluorescence of the sample to that of control cells incubated without the peptides. The binding of peptides was also expressed as EC_{50}, which is the molar concentration of the peptides producing 50% of the maximum fluorescence obtained at a concentration range between 0.001 and 100 μmol/l.

Sequential HLA-A2, HLA-B27, and a mix of HLA-B51, HLA-Cw1, and HLA-E-bound peptide pools were isolated from large quantities of either uninfected or vSC25-infected human TAP-deficient cells. These recovered peptide mixtures were subsequently separated by capillary reverse-phase HPLC and analyzed online by tandem mass spectrometry. In this analysis, two fragmentation spectra present in the vSC25-infected HLA-bound peptide pool that immunoprecipitated with the

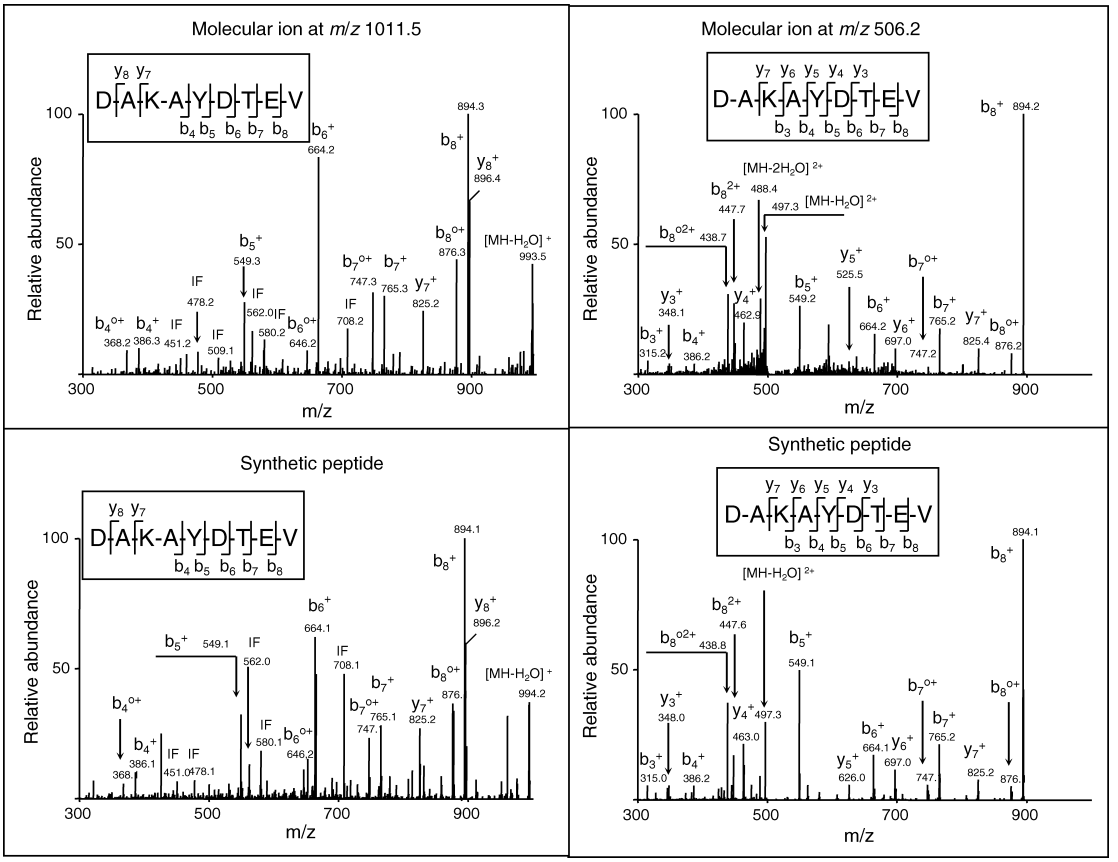

Fig. 1. Identification of the HIV ENV_{57-65} ligand in infected cell extracts by mass spectrometry. The MS/MS fragmentation spectrum was obtained after ion trap mass spectrometry of the ion peak at *m/z* 1011.5 (upper left panel) or at *m/z* 506.2 (upper right panel) of the extract of vSC25-infected cells and the corresponding synthetic peptide at *m/z* +1 (lower left panel) or at *m/z* +2 (lower right panel). The vertical axis represents the relative abundance of the parental ion and each fragmentation ion detected. The horizontal axis corresponds to the *m/z* region in which significant daughter ions were detected. Ions generated in the fragmentation are detailed, and the sequence deduced from the indicated fragments is shown in the box in each respective panel. IF indicates internal fragments of peptide fragmentation. The difference between nominal and experimentally detected monoisotopic ions was 0.001 and 0.003 Da for ion peaks at *m/z* 1011.5 and *m/z* 506.2, respectively.

W6/32 mAb but were absent in the control uninfected pool were identified at high confidence as peptides of the HIV ENV protein. Additionally, a human and viral proteome database search failed to reveal the identity of these spectra as human or vaccinia protein fragments, supporting the HIV viral origin of these sequences. The two different ion peaks at m/z 506.2 and 1011.5 corresponded to singly (Fig. 1, upper left panel) and doubly charged (Fig. 1, upper right panel) states of the peptide DAKAYDTEV, respectively. The DAKAYDTEV sequence is highly conserved between different HIV isolates (Supplementary Table 1, http://links.lww.com/QAD/A95). These peaks were assigned to the same viral amino acid sequence, which spans residues 57–65 of the HIV ENV protein. Virtually all significant fragments of both MS/MS spectra were assigned as daughter ions of the tentative peptide sequence (Fig. 1, upper panels). This theoretical assignment was confirmed on the basis of its identity with the MS/MS spectra of the corresponding synthetic peptide (Fig. 1, lower panels). No fragmentation spectra present in either HLA-A2-bound or HLA-B27-bound peptide pools were detected with sufficient confidence parameters as potential peptides of the HIV gp160 protein. Thus, these results indicate that a new TAP-independent ligand was endogenously processed and presented in the vSC25-infected cells.

Although the classic anchor motifs for HLA-A*0201 binding were described as Leu or Met at position 2 (P2) and aliphatic C-terminal residues (SYFPEITHI database, http://www.syfpeithi.de [19]), several HLA-A2-bound peptides previously described in the same database have Ala at P2 and Val C-terminal residues (for example, FASHVSPEV, EAAEVILRV, KARDPHSGHFV, KACDPHSGHFV, AAGIGILTV), which is similar to the DAKAYDTEV ligand. HLA/peptide complex stability assays were performed to confirm that the sequential immunoprecipitation was performed correctly and to exclude the possibility of residual HLA-A2-bound DAKAYDTEV ligand that was not fully immunoprecipitated with the PA2.1 (anti-HLA-A2) Ab in the first round and immunoprecipitated in the third round with the W6/32 Ab (specific for a monomorphic HLA class I determinant). Figure 1a shows that, in contrast to the control HLA-A2 ligand, the KPNA2 peptide, induction of HLA-A2 complexes with the HIV ENV_{57-65} peptide was not detected. Thus, this viral ligand does not bind to HLA-A2. The T2 human cell line also expresses HLA-B51, HLA-Cw1, and HLA-E class I molecules [8]. Therefore, to identify the HLA restriction of this ligand, new HLA/peptide complex stability assays using TAP-deficient T2 cells with specific anti-HLA-B, HLA-C, or HLA-E Abs were performed. No HLA stabilization was

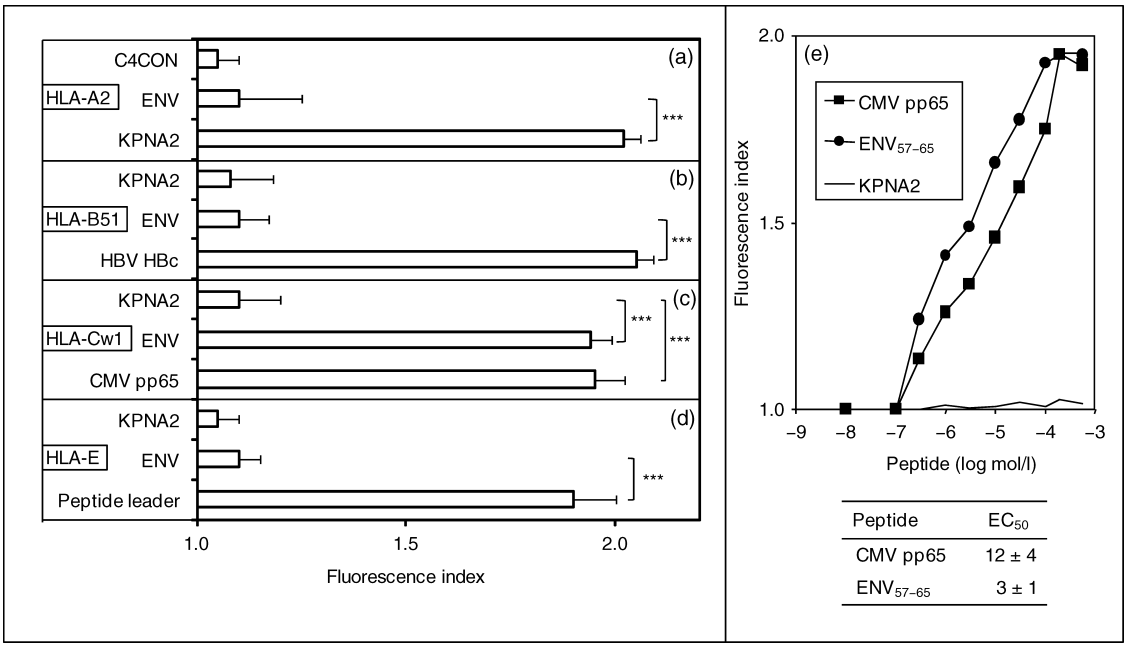

Fig. 2. Human histocompatibility complex stabilization assay with the HIV ENV_{57-65} synthetic peptide ligand. The stability of human histocompatibility complex-A2 (HLA-A2) (a), HLA-B51 (b), HLA-Cw1 (c), and HLA-E (d) at the cell surface of T2 TAP-deficient cells was measured by flow cytometry. The indicated peptides were used at 200 μmol/l. The monoclonal antibodies (mAbs) used were monoclonal PA2.1 [anti-HLA-A2, (a)], polyclonal H00003106-B01P [anti-HLA-B class I molecules, (b)], polyclonal SC-19438 [anti-HLA-C class I molecules, (c)] and monoclonal 3D12 [anti-HLA-E, (d)]. Synthetic peptides HIV ENV_{57-65} (circles), CMV $pp65_{7-15}$ (positive control, squares), and KPNA2 (negative control, single line) were titrated on cells expressing HLA-Cw1 (e), and stabilization of HLA class I molecules was measured by flow cytometry with the polyclonal SC-19438 Ab as in a–d. The results, calculated as fluorescence index (a–d) or EC_{50} (e) values ± SD, are the means of four to five independent experiments. *** Significant P values ($P < 0.0001$).

detected using either anti-HLA-B (Fig. 2b) or anti-HLA-E (Fig. 2d) Abs, indicating that the DAKAYDTEV peptide is not restricted by HLA-B51 or HLA-E class I molecules. In contrast, the numbers of HLA-peptide surface complexes induced by HIV ENV_{57-65} synthetic peptide were similar to those induced by a well known HLA-Cw1 ligand, CMV $pp85_{7-15}$ (Fig. 2c), using the anti-HLA-C Ab. The consensus peptide binding motif for HLA-Cw1 is Ala or Leu at peptide position 2 [20]. Thus, the HIV ENV_{57-65} nonamer is a natural HLA-Cw1 ligand.

Several studies have shown that peptides presented on TAP-deficient cell lines had decreased HLA binding affinity [21,22]. Thus, the relative HLA class I affinity of the DAKAYDTEV ligand was evaluated. This peptide bound to HLA-Cw1 in the range commonly found among other natural ligands. The HIV ENV_{57-65} ligand efficiently stabilized HLA-Cw1 with an EC_{50} for HLA binding of 3 ± 1 μmol/l, which is more efficient than the other optimal ligand, CMV $pp65_{7-15}$ (Fig. 2e).

A recent study defined different protease cleavage sites on HIV gp120 recognized by three major human proteases (cathepsins L, S, and D) important for antigen processing and presentation [23]. These or other uncharacterized proteases could be involved in the generation of both current HLA-Cw1 ligand and two previous TAP-independent epitopes identified in the HIV ENV protein [5,6]. These data support the hypothesis that the different cellular proteolytic systems contribute to the repertoire of presented peptides [2], thereby facilitating perhaps the immunosurveillance of infected individuals.

In summary, given that two nested TAP-independent epitopes (residues 31–40 and 37–46) were previously identified in the HIV ENV protein [5,6], the identification here of yet another HLA ligand from this protein, the HLA-Cw1 ligand between residues 57 and 65, indicates that a large fraction of at least 65 residues of gp160 is processed by different endoproteolytic cleavages, resulting in the presentation by TAP-independent pathways in different HLA class I molecules.

Acknowledgements

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Supplementary Table I Positional frequency of residues 57–65 of the HIV envelope protein and TAP-independent HIV gp160 ligand

Position	Amino acid																		HIVgp160 ligand
	A	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	X	Y	
1	0[a]	**96**	1	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	D
2	**99**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	A
3	0	0	0	0	0	0	0	**93**	0	0	3	0	0	3	0	0	0	0	0	K
4	**84**	0	0	0	5	0	0	0	2	0	0	0	0	0	7	1	0	0	0	A
5	0	0	0	0	0	8	0	0	0	0	0	0	1	0	0	3	1	0	**87**	Y
6	1	**39**	37	0	0	1	0	7	0	0	2	0	0	0	11	0	2	0	0	D
7	4	0	1	0	0	0	0	19	0	0	0	3	1	11	1	**59**	0	0	0	T
8	0	0	**100**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	E
9	19	0	0	0	0	0	1	8	0	3	0	0	2	1	2	0	**65**	0	0	V

[a]Data are expressed as percentage of relative amino acid abundance of 2331 HIV envelope sequences from Los Alamos HIV Sequence Database. The high percentage in each peptide position is bolded.

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Multiple Viral Ligands Naturally Presented by Different Class I Molecules in Transporter Antigen Processing-Deficient Vaccinia Virus-Infected Cells

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The transporter associated with antigen processing (TAP) delivers the viral proteolytic products generated by the proteasome in the cytosol to the endoplasmic reticulum lumen that are subsequently recognized by cytotoxic T lymphocytes (CTLs). However, several viral epitopes have been identified in TAP-deficient models. Using mass spectrometry to analyze complex human leukocyte antigen (HLA)-bound peptide pools isolated from large numbers of TAP-deficient vaccinia virus-infected cells, we identified 11 ligands naturally presented by four different HLA-A, HLA-B, and HLA-C class I molecules. Two of these ligands were presented by two different HLA class I alleles, and, as a result, 13 different HLA-peptide complexes were formed simultaneously in the same vaccinia virus-infected cells. In addition to the high-affinity ligands, one low-affinity peptide restricted by each of the HLA-A, HLA-B, and HLA-C class I molecules was identified. Both high- and low-affinity ligands generated long-term memory CTL responses to vaccinia virus in an HLA-A2-transgenic mouse model. The processing and presentation of two vaccinia virus-encoded HLA-A2-restricted antigens took place via proteasomal and nonproteasomal pathways, which were blocked in infected cells with chemical inhibitors specific for different subsets of metalloproteinases. These data have implications for the study of the effectiveness of early empirical vaccination with cowpox virus against smallpox disease.

The eradication of smallpox, a disease caused by variola major virus, was made possible by early empirical, cross-protective vaccination with cowpox virus and later through the massive worldwide production and administration of vaccinia virus (VACV) vaccines (18). The *Orthopoxvirus* vaccinia virus is a widely used tool for research and vaccine development (47), and recent concerns about bioterrorism and emerging infectious diseases have elicited renewed interest in VACV and other poxviruses (31). Vaccination induces a strong humoral response leading to viral clearance, and the role of cellular responses in this cross-protection is well documented (21, 56). In recent years, studies using both vaccinated humans and human histocompatibility complex (HLA)-transgenic mouse models have allowed the identification of more than 70 VACV-derived epitopes restricted by various HLA molecules (reviewed in references 30 and 32).

In cellular immunity, recognition and killing of infected cells by $CD8^+$ cytolytic T lymphocytes (CTLs) first require viral proteins to be proteolytically degraded (73). Antigen (Ag) processing generates short peptides that are translocated to the endoplasmic reticulum (ER) lumen by the transporter associated with antigen processing (TAP) and then assembled with a newly synthesized β2-microglobulin and HLA class I heavy chain. Despite initial assumptions that the multicatalytic and ubiquitous proteasome was the only protease fully capable of generating peptide ligands for presentation on HLA class I molecules, several studies have demonstrated that a growing number of alternative pathways contribute to endogenous antigen processing (reviewed in references 13 and 27).

Individuals with mutations in the TAP gene that generate nonfunctional TAP complexes have been previously described (reviewed in reference 9). Patients with this HLA class I deficiency may appear asymptomatic for long periods of their lives. TAP-deficient (TAP^-) patients do not seem particularly susceptible to viral infections or neoplasms. Therefore, their immune systems must be reasonably efficient, and antibodies (Abs), NK cells, $CD8^+$ $\gamma\delta$ T cells, and the reduced cytolytic $CD8^+$ $\alpha\beta$ T subpopulation that is specific for TAP-independent antigens may all contribute to immune defenses that protect against severe viral infections in such individuals. In addition, several strains of viruses, including cowpox virus (1), have found ways to obstruct TAP expression or function in order to prevent CTLs from identifying infected cells (reviewed in reference 36); therefore, the TAP-independent pathways must be important for killing cells infected with these viruses. The identification of TAP-independent epitopes that are conserved among orthopoxviruses could also be relevant to the study of the mechanisms of early empirical vaccination against smallpox disease as performed with cowpox virus.

Although TAP-independent viral epitopes are known (reviewed in reference 36), there has been a marked absence of methodical studies of TAP-independent epitopes and ligands restricted by different HLA molecules in cells infected with a single virus. Moreover, in all previous studies, very limited antigen processing capacity in TAP-deficient cells has been reported. There-

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TABLE 1 Specificity and activity of the inhibitors used in this study

Inhibitor	Abbreviation	Specificity	Reference(s)	Concn	% Inhibition of degradation[a]
Brefeldin A	BFA	Vesicle transport	48, 71	5 μg/ml	ND
Lactacystin	LC	Proteasome	19, 49	10 μM	ND
$(z\text{-}LL)_2$ ketone	$z\text{-}LL_2$	Signal peptide peptidase	68, 69	100 μM	13 ± 4
Leupeptin	LEU	Trypsin-like proteases and cysteine proteases	65	100 μM	38 ± 18
Pepstatin	PEPST	Aspartic proteases	34, 65	100 μM	50 ± 5
1-10 Phenanthroline	PHE	Metalloproteases and caspase-1	34, 64	50 μM	ND
Leucinethiol	LeuSH	Metalloaminopeptidases, including ERAAP	61	30 μM	ND
Benzyl succynil acid	BENZ	Metallocarboxypeptidases A and B	34	100 μM	−10 ± 8
Captopril	CAPT	ACE[b] and ACE-like proteases	34	100 μM	25 ± 2
Phosphoramidon	PHOSP	All bacterial metalloendopeptidases but few of mammalian origin	22, 34	100 μM	15 ± 4
z-VAD.fmk	z-VAD	Caspases	62	100 μM	ND[c]

[a] The activity of these inhibitors was measured as their ability to prevent proteolytic degradation in cellular extracts by the method described in reference 40. The amount of protein still present after incubation in the case of the degraded control sample was considered to represent 0% inhibition of degradation, and the nondegraded unincubated sample was taken to represent 100 % inhibition. Data represent the means of the results of two independent experiments. The negative value indicates that there was enhanced degradation in the presence of the compound. ND, not done.

[b] ACE, angiotensin-converting enzyme.

[c] The compound was found to block apoptosis (data not shown).

fore, is only one TAP-independent ligand or epitope restricted by a single HLA molecule exposed on the cell membrane surface, as suggested by these studies? Conversely, could a TAP-deficient cell simultaneously bind several viral ligands to different HLA molecules? We are interested in the identification of viral ligands presented by several common HLA antigens in TAP-deficient infected cells. Using immunoproteomic analysis, we compared HLA ligands isolated from large numbers of healthy or VACV-infected cells. This study identified 11 TAP-independent, naturally processed ligands from eight different VACV proteins in infected cells that were mostly conserved among the members of the *Orthopoxviridae* family, including cowpox virus.

MATERIALS AND METHODS

Mice. H-2 class I knockout HLA-A*0201-transgenic mice (20) were bred in our animal facilities in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals and Spanish government regulations (accreditation 28079-34A). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Institute of Health "Carlos III" (permit PI-283). All surgery was performed under conditions of sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Cell lines. T2 cells, a line of TAP-deficient human cells that express HLA-A2, HLA-B51, and HLA-Cw1 class I molecules on their surface (57), were transfected with HLA-B27 (a gift from David Yu, University of California, Los Angeles, CA). The mouse cell lines RMA (TAP positive [TAP^+]) and RMA-S (TAP^-) were transfected with HLA-A*0201 $\alpha1\alpha2$ domains, and the mouse $H\text{-}2D^b$ $\alpha3$ transmembrane and cytoplasmic domains have been previously described (52). The RMA-S transfectant cells expressing HLA-B*2705 have been previously described (66). All cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 5 μM β-mercaptoethanol (β-ME).

Synthetic peptides. Peptides were synthesized in a peptide synthesizer (model 433A; Applied Biosystems, Foster City, CA) and purified by reverse-phase high-performance liquid chromatography (HPLC). The correct molecular mass of the peptides was established by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), and their correct composition was determined by quadrupole ion trap micro-HPLC.

Inhibitors. Brefeldin A (BFA) and all protease inhibitors were purchased from Sigma-Aldrich, except for leupeptin (LEU) (Amersham-UBS), pepstatin (PEPST) (Boehringer Mannheim), Z-VAD.fmk (Enzyme System Products, Livermore, CA), $(z\text{-}LL)_2$ ketone (Merck), and lactacystin (E. J. Corey, Harvard University). The specificity and activity of all inhibitors used in this study are summarized in Table 1. For control of activity of the protease inhibitors, RMA-HLA-A*0201 cells (1×10^8) were disrupted by sonication for 15 min at 4°C and centrifuged as previously reported (40). A supernatant aliquot corresponding to 1×10^7 cells was directly frozen (nondegraded control). Equivalent aliquots were incubated in the presence of individual inhibitors at 200 μM, and digestion by cellular proteases was allowed to proceed for 5 days at 37°C in phosphate-buffered saline (PBS). Inhibitors were renewed daily. A sample incubated without inhibitors was used as the degraded control. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separation and Coomassie blue staining of these samples, the overall protein content of each lane was quantitated by densitometry using the TINA 2.09e program (Isopenmeβgeräte GmbH, Germany). Percent inhibition of protein degradation caused by each inhibitor was calculated as follows: 100 × (sample with inhibitor − degraded sample)/(nondegraded sample − degraded sample).

Infection of the T2-B27 cell line by VACV. A total of 1×10^9 to 3×10^9 T2-B27 cells were infected with VACV at a multiplicity of infection of 10 PFU/cell in 10 ml, incubated for 2 h at 37°C, and then washed. The cells were then cultured for 24 h and stained with Omnitope antiserum-fluorescein isothiocyanate (FITC), which recognizes VACV-purified virions. Samples were analyzed by fluorescence-activated cell sorting (FACS). Later, the cells were frozen.

Isolation of HLA-bound peptides. HLA-bound peptides were isolated from 4×10^{10} healthy or VACV-WR-infected T2-B27 transfectant cells. Cells were lysed in 1% CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; Sigma)–20 mM Tris-HCl buffer–150 mM NaCl (pH 7.5) in the presence of a protease inhibitor cocktail (24). HLA-peptide complexes were isolated via affinity chromatography of the soluble fraction of cell extracts with the following monoclonal antibodies (MAbs), used sequentially: PA2.1 (anti-HLA-A2) (51), ME1 (anti-HLA-B27) (14), and W6/32 (specific for a monomorphic HLA class I determinant) (6) (Fig. 1). HLA-bound peptides were eluted at room temperature with 0.1% aqueous trifluoroacetic acid (TFA), separated from the large subunits, and concentrated using a Centricon 3 column (Amicon, Beverly, MA) exactly as previously described (24).

Electrospray-ion trap mass spectrometry analysis. Peptide mixtures recovered after the ultrafiltration step were concentrated using Micro-Tip reversed-phase columns (C_{18}; Harvard Apparatus, Holliston, MA) (200

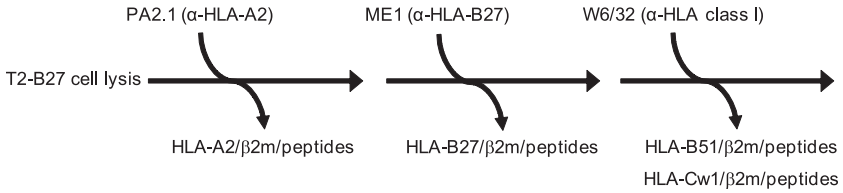


FIG 1 Diagram of sequential immunoprecipitation. Healthy or VACV-infected T2-B27 transfectant cells (4×10^{10}) were lysed. HLA-peptide complexes were isolated via affinity chromatography of the soluble fraction of cell extracts with the following mAbs, used sequentially: PA2.1 (anti-HLA-A2), ME1 (anti-HLA-B27), and W6/32 (specific for a monomorphic HLA class I determinant).

μl) (24). Each C_{18} tip was equilibrated with 80% acetonitrile–0.1% TFA, washed with 0.1% TFA, and then loaded with the peptide mixture. The tip was then washed with an additional volume of 0.1% TFA, and the peptides were eluted with 80% acetonitrile–0.1% TFA. Peptide samples were then concentrated to about 18 μl using vacuum centrifugation (24).

HLA class I peptides immunoprecipitated with each HLA-specific MAb were analyzed in three different HPLC runs by micro-liquid chromatography-tandem MS (μLC-MS/MS) using an Orbitrap XL mass spectrometer (Thermo Electron, San Jose, CA) fitted with a capillary HPLC column (Eksigent, Dublin, CA) (24). The peptides were resolved on homemade Reprosil C18 capillary columns (75 μm inner diameter) (25) with a 7 to 40% acetonitrile gradient for 2 h in the presence of 0.1% formic acid. The seven masses exhibiting the greatest intensity and single-, double-, and triple-charge states were selected for fragmentation from each full mass spectrum by collision-induced dissociation (CID).

Database searches. Sequest 3.31 software (Thermo-Fisher) (15) was used for peak-list generation of the μLC-MS/MS data. The peaks were identified by using Proteome Discoverer 1.0 SP1 software (Thermo-Fisher), combining the results obtained with Sequest 3.31 and Bioworks Browser 3.3.1 SP1 (Thermo-Fisher) (15), and using the human and virus parts of the NCBI database (January 2009), which included 656,486 proteins. The search was not limited by enzymatic specificity, the peptide tolerance was set to 0.005 Da, and the fragment ion tolerance was set to 0.5 Da (24, 43). This search was not limited by any methodological bias (selection of individual protein, use of HLA consensus-scoring algorithms, etc.). Identified peptides were selected when the following criteria were met: Sequest Xcorr $>$ 1.4 for singly, $>$ 2.2 for doubly, and $>$ 2.9 for triply charged peptides; P(pep) less than 1×10^{-3}; and mass accuracy of 0.005 Da (24, 43). When the MS/MS spectra fitted two or more peptides, only the highest-scoring peptide was analyzed. No peptides were found in a search of a reversed database. The purpose of the filtering criteria was to identify candidate vaccinia virus peptide MS/MS scans for further manual inspection to determine whether the MS/MS fragment ion fingerprint matched the identified peptide sequence. In addition, the corresponding synthetic peptide was made, and its MS/MS spectrum was used to confirm the assigned sequence.

MHC-peptide stability assays. The following synthetic peptides were used as controls in complex stability assays: KPNA2 (GLVPFLVSV, HLA-A2 restricted) (23), influenza virus NP (SRYWAIRTR, HLA-B27 restricted) (67), hepatitis B virus (HBV) HBc_{19-27} (LPSDFFPSV, HLA-B51 restricted) (8), cytomegalovirus (CMV) $pp65_{7-15}$ (RCPEMISVL, HLA-Cw1 restricted) (33), and C4CON (QYDDAVYLK, HLA-Cw4 restricted) (17). Either RMA-S transfectants or T2 cells expressing small amounts of major histocompatibility complex (MHC) class I on the cell surface were incubated at 26°C for 16 h in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). This allowed the expression of empty MHC class I molecules (without antigenic peptide) that are stable only at 26°C, and not at 37°C, on the cell membrane. The cells were washed and incubated for 2 h at 26°C with various concentrations of peptide in the same medium. Afterward, the cells were kept at 37°C and collected after 4 h for flow cytometry analysis. This assay allows for internalization of empty MHC class I molecules and can therefore discriminate between bound and unbound peptides. HLA expression levels were measured using monoclonal PA2.1 (anti-HLA-A2), monoclonal ME1 (anti-HLA-B27), polyclonal H00003106-B01P (specific for HLA-B class I molecules) (Abnova, Taipei, Taiwan), and polyclonal SC-19438 (specific for HLA-C class I molecules) (Santa Cruz Biotechnology, Santa Cruz, CA) Abs as previously described (41). Samples were acquired on a FACSCanto flow cytometer (BD Biosciences, San Jose, CA) and analyzed using CellQuest Pro 2.0 software (BD Bioscience). Cells incubated without peptides had peak fluorescence intensities close to those of background staining with secondary Ab alone. The fluorescence index (FI) was calculated as the ratio of the mean channel fluorescence of the sample to that of control cells incubated without the peptides. The binding of peptides was also expressed as the 50% effective concentration (EC_{50}), which is the molar concentration of the peptides producing 50% of the maximum fluorescence obtained at a concentration range between 0.001 and 100 μM.

Magnetic antigen cell separation (MACS). Mouse T lymphocytes were isolated by the depletion of non-T CD8$^+$ cells (negative selection) and the use of a T CD8a$^+$ cell isolation kit (Miltenyi Biotec GmbH, Gladbach, Germany) according to the manufacturer's specifications. The purity of the cell preparations recovered after negative selection was verified by fluorescence-activated cell sorting (FACS) and found to be higher than 95% for CD8$^+$ T lymphocytes (3).

***Ex vivo* ICS.** Intracellular cytokine staining (ICS) assays were performed as described previously (58). Purified CD8$^+$ T lymphocytes were obtained from HLA-A*0201-transgenic mice up to 30 days (memory) postintraperitoneal (post-i.p.) infection with 1×10^7 PFU of VACV-WR, stimulated for 2 h with either RMA or RMA-S HLA-A*0201 cells infected with VACV-WR, and incubated overnight in the presence of BFA (5 μg/ml). Later, cells were incubated with FITC-conjugated anti-CD8 MAb (ProImmune, Oxford, United Kingdom) for 30 min at 4°C, fixed with Intrastain kit (DakoCytomation, Glostrup, Denmark) reagent A, and incubated with phycoerythrin (PE)-conjugated anti-gamma interferon (IFN-γ) MAb (BD PharMingen, San Diego, CA) in the presence of Intrastain kit permeabilizing reagent B for 30 min at 4°C. Events were acquired and analyzed as described for the MH-peptide stability assays.

T cell lines, cytotoxicity assays, and ICS. Polyclonal SIINFEKL or VACV peptide-monospecific CTLs were generated by immunizing mice i.p. with 1×10^7 PFU of VACV-$OVA_{257-264}$ (encoding the miniprotein MSIINFEKL) or VACV-WR, respectively, as previously described (40, 45). Splenocytes from immunized mice were restimulated *in vitro* with mitomycin C-treated spleen cells pulsed with a 10^{-6} M concentration of the respective peptide and cultured in alpha-minimal essential medium (α-MEM) supplemented with 10% FBS–1×10^{-7} M peptide–1% 2-ME. Recombinant human interleukin-2 for the long-term propagation of peptide-specific CTL lines was generously provided by Hoffmann-LaRoche. The RMA-HLA-A*0201 cells were used as target cells in standard 4-h cytolytic assays (40).

ICS assays to detect the recognition of infected cells by polyclonal CTL cell lines were performed as previously described (10). CTL lines were stimulated for 4 h in the presence of BFA (5 μg/ml) and target cells that had been infected overnight with VACV or VACV-$OVA_{257-264}$. When protease inhibitors were used, all drugs were added 15 min before the virus and kept at a 5-fold-higher concentration during the 1-h adsorption

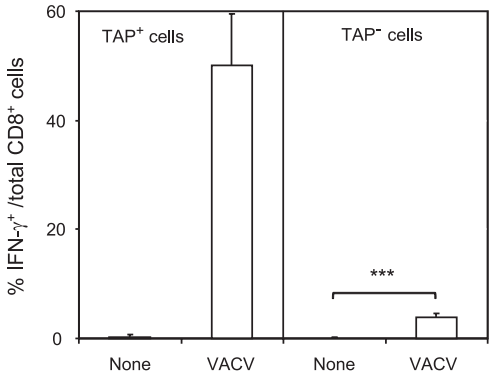


FIG 2 Recognition of TAP$^+$ and TAP$^-$ cell lines by VACV-specific CD8$^+$ T lymphocytes. HLA-A*0201 TAP$^+$ cells (RMA; left panel) and TAP$^-$ cells (RMA-S; right panel) were infected with VACV at a multiplicity of infection of 40 PFU/cell and analyzed by ICS for CD8$^+$ T cell activation. The results are calculated as the means ± standard deviations (SD) of the results of three or four independent experiments. ***, $P < 0.0001$.

period than throughout the infection. After the virus inoculum was washed, the inhibitors were kept at the concentrations indicated for the individual experiments. The inhibitors were not toxic at the indicated concentrations, since they affected neither antigen presentation by either of the $A10L_{688-696}$ and $A17L_{9-17}$ epitopes (see below) or VACV infection when the Omnitope antiserum with specificity for VACV proteins from purified virions (ViroStat Inc., Portland, ME) was used (see Fig. S1 in the supplemental material). The ICS was performed with polyclonal CTL as described for *ex vivo* ICS.

***In vivo* cytotoxicity assay.** *In vivo* cytotoxicity assays were performed as previously published (45). Spleens were obtained, erythrocytes were removed, and HLA-A*0201 splenocytes were split into two populations and labeled with either a high concentration (5 μM) or a low concentration (0.5 μM) of carboxyfluorescein succinimidyl ester(CFSE). After excess CFSE was removed by washing, $CFSE^{high}$ spleen cells were pulsed with 10^{-6} M VACV peptides for 30 min at 37°C. Excess peptide was washed at least twice, and $CFSE^{high}$ peptide-pulsed cells were mixed with equal numbers of $CFSE^{low}$ cells. A total of 8×10^6 cells of the spleen cells mixed suspension were i.p. injected into each HLA-A*0201-transgenic mouse that had been left uninfected or had been i.p. infected with VACV-WR (1×10^7 PFU) 7 days earlier. Two days later, the peritoneal cavity was subjected to lavage, spleens were extracted, and the cells were analyzed by flow cytometry using a FACSCanto flow cytometer to measure *in vivo* killing. Data were analyzed using CellQuest Pro 2.0 software. Specific lysis was calculated as previously published (45) according to the following formula: [1 − (ratio unprimed/ratio primed) × 100], where the ratio unprimed values represent % $CFSE^{low}$/% $CFSE^{high}$ cells remaining in control uninfected recipients and the ratio primed values represent % $CFSE^{low}$/% $CFSE^{high}$ cells remaining in experimentally infected recipients.

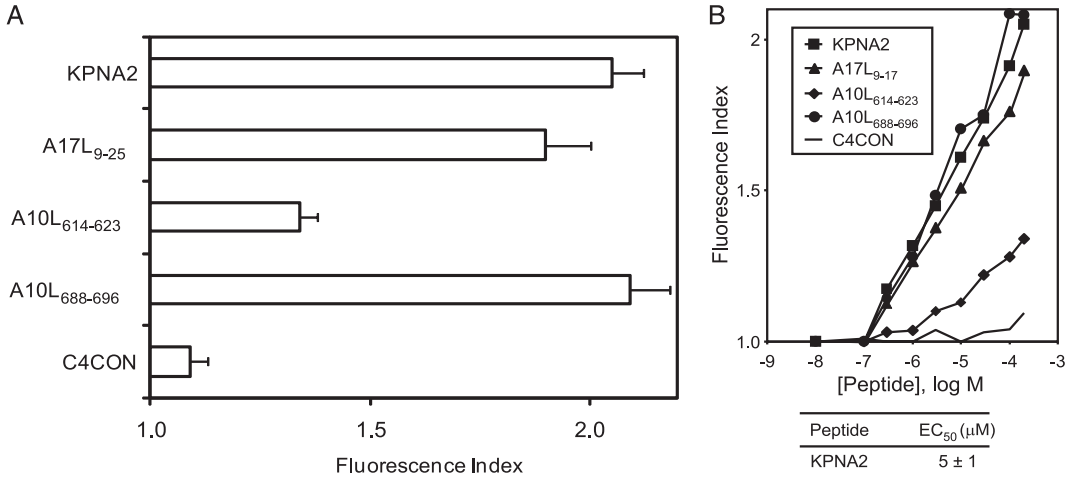
RESULTS

VACV-specific CD8$^+$ T cells recognize TAP-deficient HLA-A*0201-transfected cells. As the first step in the study of TAP-independent HLA-restricted responses to vaccinia virus, HLA-A*0201-transgenic mice were immunized with the virus. Next, the VACV-specific CD8$^+$ response was evaluated using intracellular cytokine staining (ICS) assays. A strong *ex vivo* response (i.e., 50.1% ± 9.4% of CD8$^+$ cells secreted IFN-γ) specific for this virus was detected in TAP-positive (TAP$^+$) target cells (Fig. 2, left panel). Additionally, a small fraction of VACV-specific CD8$^+$ T lymphocytes recognized infected TAP-deficient cells (3.8 ± 0.8% of CD8$^+$ cells secreted IFN-γ) (Fig. 2, right panel). These data indicate the existence of a TAP-independent antigen-processing pathway(s) of some vaccinia virus epitopes in infected cells that could be recognized by specific CD8$^+$ T lymphocytes.

Physiological processing generates three different viral HLA-A2 ligands in human TAP-deficient vaccinia virus-infected cells. HLA-A2-bound peptide pools were isolated from large numbers of either healthy or VACV-infected human TAP-deficient cells (110 ± 20 mean fluorescence intensity [MFI] in VACV-infected cells versus 15 ± 5 in healthy cells stained with an anti-VACV antiserum). These peptide mixtures were subsequently separated by reverse-phase HPLC and analyzed by mass spectrometry. According to the results observed with bioinformatics tools, three fragmentation spectra present in the VACV-infected HLA-A2-bound peptide pool, but absent from the control uninfected pool, were resolved with high-confidence parameters as peptides of vaccinia virus proteins. A human proteome database search failed to identity any of these spectra as human protein fragments, confirming the viral origin of these peptides. The first ion peak, with an *m/z* of 926.4, was assigned to the viral amino acid sequence MLDDFSAGA, spanning residues 9 to 17 of the A17L protein of vaccinia virus (see Fig. S2 in the supplemental material, upper panel). In addition, two different ion peaks at *m/z* 974.6 and 514.8 were assigned to peptides of the same viral protein. These ion peaks corresponded to the SPEGE ETII peptide (see Fig. S2 in the supplemental material, middle panel) and ILDRIITNA peptide (see Fig. S2 in the supplemental material, lower panel), which span residues 614 to 623 and residues 688 to 696, respectively, of the A10L protein. Virtually all significant fragments of the three MS/MS spectra were assigned as daughter ions of the putative peptidic sequences (see Fig. S2 in the supplemental material). This theoretical assignment was confirmed by determination of identity with the MS/MS spectrum of the corresponding synthetic peptide (see Fig. S2 in the supplemental material). Therefore, these results indicate that a total of three TAP-independent HLA-A2 ligands were endogenously processed and presented in the VACV-infected cells.

Binding affinity of TAP-independent vaccinia virus ligands for the A*0201 molecule. The classical anchor motifs for HLA-A*0201 binding, Leu or Met at position 2 (P2) and aliphatic C-terminal residues (SYFPEITHI database; http://www.syfpeithi.de [54]), were present in two of the three TAP-independent viral ligands detected. In contrast, the $A10L_{614-623}$ ligand presented Pro at P2, although it coimmunoprecipitated with an HLA-A2-specific MAb and thus could have represented an unusual HLA-A2-restricted ligand. To confirm that HLA-A*0201 was the MHC class I molecule that presented these ligands, MHC-peptide complex stability assays were performed using TAP-deficient RMA-S cells transfected with the HLA-A*0201 molecule (Fig. 3). The two viral ligands with HLA-A2 anchor motifs, $A10L_{688-696}$ and $A17L_{9-17}$, bound to HLA-A*0201 class I molecules with EC_{50}s in the range commonly found among other natural high-affinity ligands (Fig. 3B). In contrast, HLA affinity for the $A10L_{614-623}$ ligand was substantially lower, as the absence of HLA-A2 anchor motifs suggested; therefore, this peptide must be considered a low-affinity ligand. These data confirm that all ligands detected in vaccinia virus-infected cells were endogenously presented in association with the A*0201 molecule.

Three viral HLA-B*2705 ligands were endogenously processed in human TAP-deficient vaccinia virus-infected cells. To date, about 60 human TAP-independent MHC class I ligands are



Peptide	EC_{50} (μM)
KPNA2	5 ± 1
$A17L_{9-17}$	10 ± 5
$A10L_{614-623}$	> 100
$A10L_{688-696}$	4 ± 2

FIG 3 HLA-A*0201 stabilization assay with synthetic VACV ligands. (A) The stability of HLA-A*0201-peptide complexes on the surface of RMA-S transfectant cells was measured by flow cytometry. The indicated peptides were used at 200 μM. The mAb used was PA2.1. (B) The titration curves for synthetic VACV $A17L_{9-17}$, $A10L_{614-623}$, and $A10L_{688-696}$ peptides with HLA-A*0201 are depicted. The C4CON and KPNA2 peptides were used as negative and positive controls, respectively. The results, calculated as fluorescence index values (see panel A), represent the means of the results of three or four independent experiments. The calculated EC_{50}s ± SD are shown in panel B.

known (36, 70), and these are mostly restricted by HLA-A2 and derived from the cleavage of signal sequences generated by the signal peptidase (SPase) complex. The A*0201-restricted CTL epitopes of vaccinia virus that we detected in TAP-deficient cells could therefore be exceptional, and TAP-independent antigen-processing pathways might be unable to generate vaccinia virus peptides that could bind to other HLA class I molecules. HLA-B27, which was previously described as an allele with high TAP dependency (2), was used to obtain peptide pools from either healthy or VACV-infected TAP-deficient cells, as used to identify the HLA-A*0201 ligands. Again, three fragmentation spectra present in the VACV-infected, HLA-B27-bound peptide pool, but absent from the control uninfected pool, were also resolved as peptides of vaccinia virus proteins. The human proteome database search failed to identify these spectra as representing human protein fragments, confirming the viral origin of these HLA-B27-bound peptides. The first ion peak, with an *m/z* of 428.8, was assigned to the viral amino acid sequence SRGYFEHMKK, which spans residues 867 to 876 of the A10L protein of vaccinia virus (see Fig. S3 in the supplemental material, upper panel), indicating that antigen processing of this protein could generate several viral ligands bound to two different HLA class I molecules. The second ion peak, at *m/z* 567.6, was assigned to the peptidic sequence YR LQGFTNAGIVAYK (see Fig. S3 in the supplemental material, middle panel), which spans residues 16 to 30 of the K2L protein. Finally, the third ion peak, at *m/z* 471.8, corresponded to a WQT MYTN peptide (see Fig. S3 in the supplemental material, lower panel), which spans residues 53 to 59 of the B8R protein. Figure S3 in the supplemental material shows that all significant fragments of these three MS/MS spectra were assigned as daughter ions of the putative peptidic sequences. As HLA-A2 ligands, these assignments were confirmed by identity with the MS/MS spectrum of the corresponding synthetic peptide (see Fig. S3 in the supplemental material). In addition, the $K2L_{16-30}$ ligand was also identified as a molecular ion at *m/z* +2 (see Fig. S5 in the supplemental material). Collectively, these results indicate that similar numbers of TAP-independent ligands were endogenously processed and presented by HLA-A2 and HLA-B27 class I molecules in the same vaccinia virus-infected cells.

Binding affinity of TAP-independent vaccinia virus ligands for the B*2705 molecule. The $A10L_{867-876}$ and $K2L_{16-30}$ (but not $B8R_{53-59}$) peptides have the known anchor motifs for HLA-B*2705 binding of Arg at P2 and basic or aliphatic C-terminal residues (SYFPEITHI database [54]). As the $B8R_{53-59}$ peptide was also coimmunoprecipitated with an HLA-B27-specific MAb, it could be an unusual HLA-B27-restricted ligand. To confirm that HLA-B*2705 is the MHC class I molecule that presents these ligands, MHC-peptide complex stability assays were performed using TAP-deficient RMA-S cells transfected with the HLA-B*2705 molecule (Fig. 4). The two viral ligands with HLA-B27 anchor motifs, $A10L_{867-876}$ and $K2L_{16-30}$, bound to HLA-B*2705 class I molecules with EC_{50}s to similar those of other natural high-affinity ligands (Fig. 4B). In contrast, the HLA affinity was substantially lower for the $B8R_{53-59}$ ligand, as suggested by the absence of HLA-B27 anchor motifs, and this peptide could be considered a low-affinity ligand. These data confirm that the ligands detected in vaccinia virus-infected cells were endogenously presented in association with the B*2705 molecule. In summary, either HLA-A*0201 or B*2705 class I molecules can bind both high- and low-affinity ligands derived from different vaccinia virus proteins.

Six vaccinia virus ligands were endogenously presented by HLA-B51 and/or HLA-Cw1 class I molecules in human TAP-deficient cells. Given that several TAP-independent viral ligands were identified in association with either HLA-A*0201 or B*2705 class I molecules (see above), we investigated the possibility of new vaccinia virus ligands being presented by other HLA class I molecules expressed in the same T2 cells. As in the previous two analyses, six fragmentation spectra were found in the VACV-infected

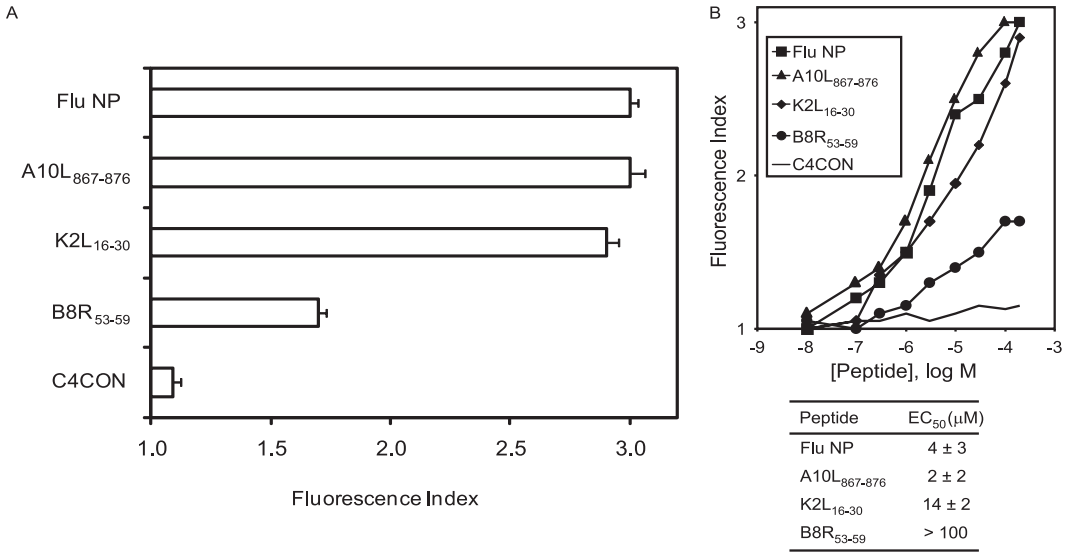
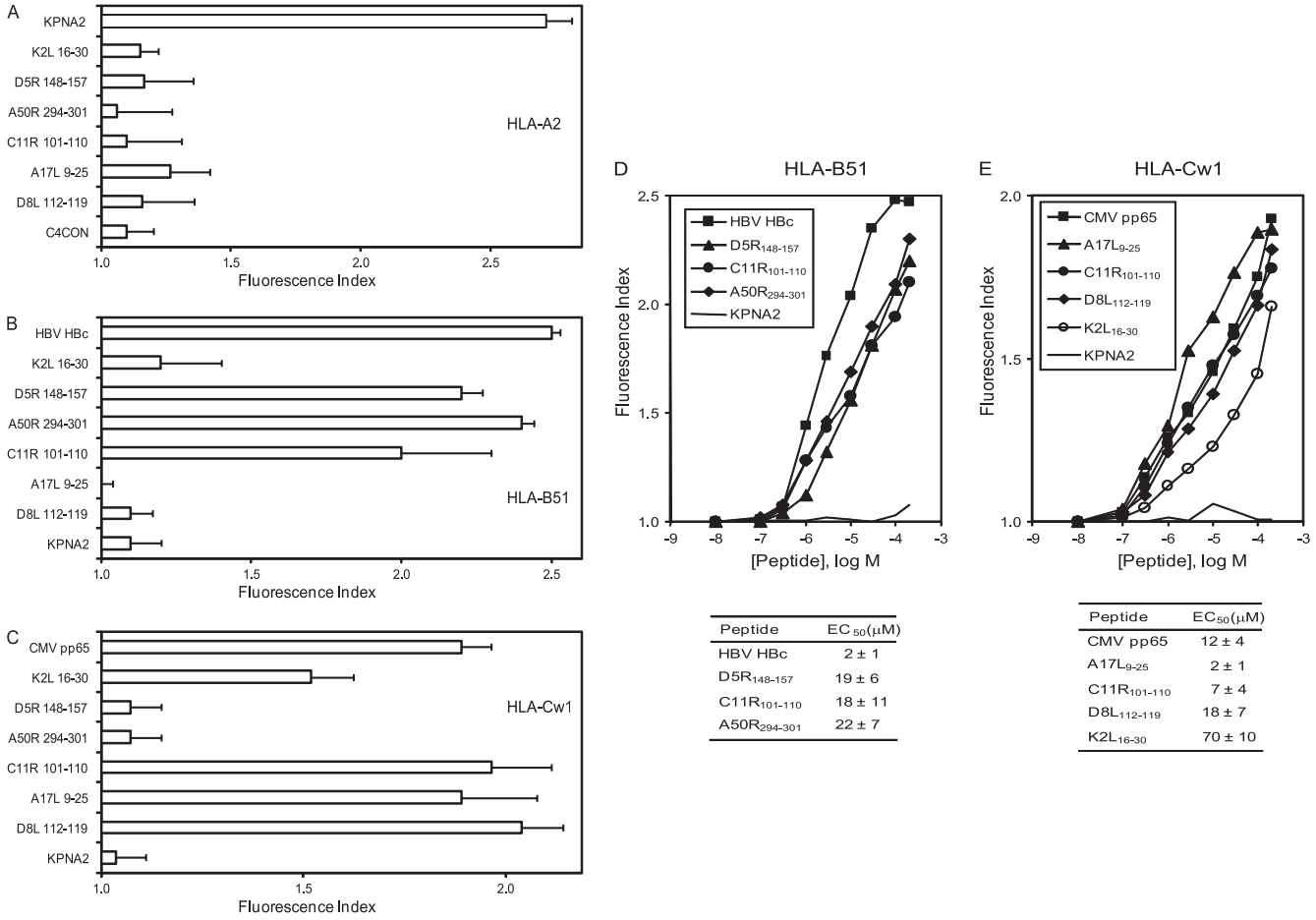


FIG 4 HLA-B*2705 stabilization assay with synthetic VACV ligands. (A) Stability of HLA-B*2705-peptide complexes on the surface of RMA-S transfectant cells measured by flow cytometry. The indicated peptides were used at 200 μM. The mAb used was ME1. (B) The titration curves for synthetic VACV A10L$_{867-876}$, K2L$_{16-30}$, and B8R$_{53-59}$ peptides with HLA-B*2705 are depicted. The C4CON and influenza virus (Flu) NP peptides were used as negative and positive controls, respectively. The results, as in Fig. 2, represent the means of the results of four independent experiments.

peptide pool that were absent from the uninfected control pool, and these spectra were also resolved as peptides of vaccinia virus proteins. Furthermore, a human proteome database search also failed to identify these spectra as human protein fragments, confirming the viral origin of these HLA-bound peptides. Supplemental Fig. S4 and S5 show the experimentally obtained MS/MS spectra and their respective assignments. Each putative peptidic sequence was confirmed according to its identity with the MS/MS spectrum of the corresponding synthetic peptide (see Fig. S4 and S5 in the supplemental material). Five of these peptides, IAMKR TLLEL (D5R$_{148-157}$), LPFGSLGI (A50R$_{294-301}$), IPSPGIMLV (C11R$_{101-110}$), MLDDFSAGAGVLDKDL (A17L$_{9-25}$), and DGLI IISI (D8L$_{112-119}$), represented new viral sequences. Surprisingly, the K2L$_{16-30}$ ligand previously detected in association with HLA-B*2705 (see Fig. S3 in the supplemental material) was also immunoprecipitated in the third round with the W6/32 Ab. As HLA-A2, HLA-B51, and HLA-Cw1 present peptides with similar anchor motifs (SYFPEITHI database [54]), HLA-peptide complex stability assays were performed to confirm that the sequential immunoprecipitation was performed correctly and to exclude the possibility that residual HLA-A2-bound ligands that were not fully immunoprecipitated with the PA2.1 (anti-HLA-A2) Ab in the first round would be immunoprecipitated in the third round with the W6/32 Ab (specific for a monomorphic HLA class I determinant). Figure 5A shows that, in contrast to the positive-control KPNA2 peptide results, binding of HLA-A2 complexes to these six ligands was not detected. Therefore, these viral ligands do not bind to HLA-A2, which validates the experimental strategy used. In addition, to identify the HLA restriction of these ligands, new HLA-peptide complex stability assays using TAP-deficient T2 cells with specific anti-HLA-B or anti-HLA-C Abs were performed. The numbers of HLA-peptide surface complexes induced by the IAM KRTLLEL (D5R$_{148-157}$), LPFGSLGI (A50R$_{294-301}$), and IPSPG IMLV (C11R$_{101-110}$) synthetic peptides were similar to those induced by a well-known HLA-B51 ligand, HBV HBc$_{19-27}$ (Fig. 5B), by the use of the anti-HLA-B Ab, indicating that these peptides were restricted by the HLA-B51 class I molecule. HLA stabilization was detected using anti-HLA-C (Fig. 5C) Ab with IPSPG IMLV (C11R$_{101-110}$), MLDDFSAGAGVLDKDL (A17L$_{9-25}$), and DGLIIISI (D8L$_{112-119}$), indicating that these peptides were restricted by the HLA-Cw1 allele. In addition, the K2L$_{16-30}$ ligand also bound to HLA-B*2705 (see Fig. S3 in the supplemental material and Fig. 4) presented efficient binding to HLA-Cw1 class I molecule, justifying their dual immunoprecipitation (Fig. 5). Five of these six viral ligands bound to HLA-B51 (Fig. 5D) or HLA-Cw1 (Fig. 5E) class I molecules with EC$_{50}$s similar to those of other natural high-affinity ligands. The only exception was the K2L$_{16-30}$ peptide, which showed a substantially lower affinity for HLA-Cw1, indicating that this peptide could be considered a low-affinity ligand (Fig. 5E).

Thirteen natural peptide-HLA class I complexes were formed simultaneously in the same infected TAP-deficient cells. A total of 11 viral ligands that bound to the four HLA class I molecules expressed in the T2 cells were identified (summarized in Table 2). Similar numbers (3 or 4) of ligands were identified in association with HLA class I molecules previously described as showing low (HLA-A2), high (HLA-B27), or unknown (HLA-B51 and HLA-Cw1) TAP dependency. The same N-terminal core peptide, MLDDFSAGA, was found in two different ligands, A17L$_{9-17}$ and A17L$_{9-25}$, bound to HLA-A2 and HLA-Cw1, respectively. Ten sequences represented new vaccinia HLA ligands. Most were restricted by a single HLA allele, but two ligands, K2L$_{16-30}$ and C11R$_{101-110}$, were found to be associated with two different alleles each, alleles HLA-B27 and HLA-Cw1 and alleles HLA-B51 and HLA-Cw1, respectively. This implies that 13 different natural peptide-HLA class I complexes were formed simultaneously in the same infected TAP-deficient cells.

Conservation of ligands among members of the *Orthopoxvirus* family. The sequences of 11 vaccinia virus ligands identified in the WR strain were compared with homologs derived from vari-



Peptide	EC_{50}(μM)
HBV HBc	2 ± 1
$D5R_{148-157}$	19 ± 6
$C11R_{101-110}$	18 ± 11
$A50R_{294-301}$	22 ± 7

Peptide	EC_{50}(μM)
CMV pp65	12 ± 4
$A17L_{9-25}$	2 ± 1
$C11R_{101-110}$	7 ± 4
$D8L_{112-119}$	18 ± 7
$K2L_{16-30}$	70 ± 10

FIG 5 HLA class I stabilization assay of synthetic VACV ligands. (A, B, and C) The stability of HLA-A2 (A), HLA-B51 (B), and HLA-Cw1 (C) at the cell surface of T2 TAP-deficient cells was measured by flow cytometry. The indicated peptides were used at 200 μM. (D and E) Titration curves for the indicated synthetic VACV peptides, immunoprecipitated with W6/32 Ab, with HLA-B51 (D) or HLA-Cw1 (E). The KPNA2 peptide was used as negative control (solid line). The HBV HBc_{19-27} and CMV $pp65_{7-15}$ peptides were used as positive controls for binding to the HLA-B51 and HLA-Cw1 alleles, respectively. The Abs used were monoclonal PA2.1 (anti-HLA-A2; see panel A), polyclonal H00003106-B01P (anti-HLA-B class I molecules; see panels B and D), and polyclonal SC-19438 (anti-HLA-C class I molecules; see panels C and E). The results, as in Fig. 2, represent the means of the results of four to six independent experiments.

ous poxviruses. This comparison included the Copenhagen and MVA strains of VACV, two strains of human poxvirus (variola major and variola minor), and other mammalian poxviruses, such as camelpox, cowpox, ectromelia virus, horsepox, monkeypox, rabbitpox, and taterapox. This study revealed a high degree of conservation of the ligands among orthopoxviruses (Table 3). Ten of these 11 ligands are almost fully conserved in the variola major and minor viruses, with a minor substitution in the P7 position of the $C11R_{101-110}$ sequence. Only 60% of the previously described TAP^+ vaccinia virus epitopes are conserved in the variola proteome (46, 50, 53), indicating that TAP-independent vaccinia ligands are more highly conserved between immunogenic and pathological poxviruses than TAP-dependent epitopes.

Low hydrophobicity in TAP-independent vaccinia virus ligands. Eight TAP-independent epitopes, restricted by four different HLA class I molecules, were previously characterized with respect to their Epstein-Barr virus (EBV) CTL response (reviewed in reference 38). For this virus, only peptides from the BRLF1 and LMP2 proteins with high hydrophobicity are TAP independent (37, 38). Therefore, we tested this correlation in our vaccinia virus system. The hydrophobicity values for all TAP-independent EBV epitopes were determined over a range that reached a maximum of 2.1 on the grand average of hydropathicity (GRAVY) scale, with the maximum value found for the EBV LMP2 LLWTLVVLL peptide (Table 4). All three HLA-B*2705 ligands and the $A10L_{614-623}$ HLA-A*0201-restricted ligand identified in our study were hydrophilic, with negative GRAVY values (Table 4). Only the vaccinia virus HLA-Cw1-restricted $D8L_{112-119}$ epitope, with a GRAVY value of 2.1, was hydrophobic. The other six ligands showed low positive GRAVY values, indicating low hydrophobicity (Table 4). The GRAVY mean value for these 11 vaccinia ligands was only 0.3, which was very different from the value for the EBV epitopes and similar to the value for the TAP-dependent vaccinia epitopes (Table 4). These results show that hydrophobicity is not a necessary condition for the TAP-independent presentation of ligands and epitopes from other viruses such as vaccinia virus.

Recognition of HLA-A*0201 ligands by specific $CD8^+$ T cells in HLA-transgenic mice immunized with vaccinia virus. To study the immunogenicity of the identified HLA-A*0201 viral ligands, transgenic HLA-A*0201-positive mice were immunized with VACV. Later, physiological measurement of the functional *in vivo* activity of $CD8^+$ T lymphocytes against HLA-A2 viral ligands

TABLE 2 Summary of HLA molecules bound by vaccinia virus epitopes

Ligand	Sequence	MAb used[a]	Molecule detection result[b]			
			HLA-A2	HLA-B27	HLA-B51	HLA-Cw1
$A17L_{9-17}$	MLDDFSAGA	PA2.1	+[c]	ND	ND	ND
$A10L_{614-623}$	SPEGEETII	PA2.1	+	ND	ND	ND
$A10L_{688-696}$	ILDRIITNA	PA2.1	+	ND	ND	ND
$A10L_{867-876}$	SRGYFEHMKK	ME1	ND	+	ND	ND
$B8R_{53-59}$	WQTMYTN	ME1	ND	+	ND	ND
$K2L_{16-30}$	YRLQGFTNAGIVAYK	ME1/W6-32	−	+	−	+
$D5R_{148-157}$	IAMKRTLLEL	W6-32	−	ND	+	−
$A50R_{294-301}$	LPFGSLGI	W6-32	−	ND	+	−
$C11R_{101-110}$	IPSPGIMLV	W6-32	−	ND	+	+
$A17L_{9-25}$	MLDDFSAGAGVLDKDL	W6-32	−	ND	−	+
$D8L_{112-119}$	DGLIIISI	W6-32	−	ND	−	+

[a] The MAbs used for the sequential immunoprecipitations were PA2.1 (specific for HLA-A2), ME1 (specific for HLA-B27), and W6-32 (specific for a monomorphic HLA-A, HLA-B, or HLA-C determinant).
[b] ND, not done.
[c] Significant difference ($P < 0.001$) compared with the negative control results.

as identified by mass spectrometry was carried out. HLA-A*0201-transgenic mice eliminated VACV peptide-pulsed $CFSE^{high}$ cells when they had been previously immunized but not when uninfected control mice were used (Fig. 6). In addition, up to 30 days postimmunization, polyclonal CTL lines were generated that were monospecific for each HLA-A2 viral ligand identified by mass spectrometry. These CTL lines specifically recognized peptide-pulsed cells (Fig. 7), indicating that the HLA ligands were all A*0201-restricted CTL epitopes and that they were simultaneously recognized as part of the long-term memory response to vaccinia virus. Also, these $CD8^+$ effector lines specifically recognized VACV-infected cells (Fig. 7D).

Several attempts to induce CTL responses to the identified viral B*2705-restricted ligands in HLA-B*2705-transgenic mice were unsuccessful. This was also the case for the influenza NP epitope previously described in a study of influenza virus-infected $B27^+$ humans (67) that we used as a CTL-positive control. The mouse model used carried HLA-B27 and endogenous murine H2 class I molecules (28), in contrast to the HLA-A2-transgenic mouse knockout used previously for these mouse molecules (52). This may have reduced the efficiency of HLA-restricted Ag-specific responses to undetectable levels (11), although the possibility that the T cell repertoire was a limiting factor could not be discounted. Unfortunately, HLA-B27-transgenic mice deficient for H2 class I expression (11) were not available. Therefore, the study of HLA-B*2705-restricted CTL responses to vaccinia virus ligands was not feasible.

Three different antigen-processing pathways were involved in the presentation of $A10L_{688-696}$ and $A17L_{9-17}$ epitopes in infected TAP-sufficient cells. To study the antigen-processing pathways involved in the endogenous generation of the $A10L_{688-696}$ and $A17L_{9-17}$ viral epitopes, we investigated the presentation of these epitopes to specific CTLs in the presence of diverse protease inhibitors in VACV-infected TAP-sufficient cells.

First, to demonstrate that these HLA-A2-restricted epitopes required endogenous processing, their presentation was analyzed in the presence of BFA. This drug blocks class I export beyond the *cis*-Golgi compartment (39), thus preventing the surface expression of newly assembled HLA class I-peptide complexes from an endogenous origin (Table 1 summarizes the specificity of all inhibitors used). The complete inhibition of specific lysis in both specific CTL lines caused by the addition of BFA during vaccinia infection (Fig. 8A) demonstrated that the two relevant epitopes were indeed generated from proteins endogenously processed in infected cells.

Lactacystin, a *Streptomyces* metabolite (19, 49), was used to study the involvement of the proteasome in the presentation of these epitopes. This drug had no effect on the specific recognition

TABLE 3 Conservation of viral HLA ligands in several orthopoxviruses[a]

Poxvirus	**$A17L_{9-17}$ (HLA-A2)**	**$A10L_{614-623}$ (HLA-A2)**	**$A10L_{688-696}$ (HLA-A2)**	**$A10L_{867-876}$ (HLA-B27)**	**$B8R_{53-59}$ (HLA-B27)**
VACV WR	MLDDFSAGA	SPEGEETII	ILDRIITNA	SRGYFEHMKK	WQTMYTN
VACV Copenhagen	---------	---------	---------	----------	-------
VACV MVA	---------	---------	---------	----------	-------
Variola major	---------	---------	---------	----------	-------
Variola minor	---------	---------	---------	----------	-------
Camelpox	---------	---------	---------	----------	-------
Cowpox	---------	---------	---------	----------	-------
Ectromelia virus	---------	---------	---------	----------	-------
Horsepox	---------	---------	---------	----------	-------
Monkeypox	---------	---------	---------	----------	-------
Rabbitpox	---------	---------	---------	----------	-------
Taterapox	---------	---------	---------	----------	-------

[a] The sequences used were obtained from the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

by $A10L_{688-696}$- or $A17L_{9-17}$-specific CTLs of target cells infected with VACV (Fig. 8A). Although the proteasome may be involved in the antigen processing of these epitopes, these data suggest that the lactacystin-inhibitable proteasome activity is not absolutely required.

Because several endogenous TAP-independent HLA-2 class I ligands are derived from cleavage of the signal sequences generated by the signal peptidase (SPase) complex (36, 70) and because no specific inhibitor of this enzymatic activity was available, the involvement of the SPase complex in $A17L_{9-17}$ ligand production could not be studied directly. SPase-processed peptides need further cleavage by signal peptide peptidase (SPPase) (42, 68, 69). Therefore, the involvement of SPPase in antigen presentation was tested by treating target cells with the SPPase-specific inhibitor $(z\text{-}LL)_2$ ketone (68, 69). In similarity to lactacystin results, the inhibition of specific presentation was not detected with this drug (Fig. 8A).

To identify proteases distinct from the proteasomes that could contribute to antigen processing of both HLA-A2 epitopes, experiments with several specific protease inhibitors were performed. Leupeptin (LEU) (65), pepstatin (PEPST) (34, 65) and 1,10-phenanthroline (PHE) (34, 64) were initially tested because they specifically inhibit different protease families (Table 1), thereby covering a wide range of proteases. Figure 8A shows that these three inhibitors had no effect on the specific recognition of target cells infected with VACV. In addition, because the activity of ERAAP, an enzyme involved in antigen processing (59, 72), is not fully blocked by PHE at the concentration used in this study, leucinethiol (LeuSH) (Table 1) (61) was also included. Like the other protease inhibitors, LeuSH did not inhibit the recognition of infected cells (Fig. 8A), indicating that ERAAP cannot be involved in the generation of these TAP-independent epitopes.

In summary, the inhibitors used did not block the presentation of the two viral epitopes tested. The two most likely explanations for these results are as follows. First, these epitopes could have been processed by a protease(s) that was not blocked by the collection of inhibitors used in Fig. 8A. This explanation is not likely, because the inhibitors were chosen to cover a wide range of protease classes. Alternatively, these epitopes could have been independently processed in parallel by different proteases, meaning that the different antigen-processing pathways would need to be inhibited at the same time to produce an effect. To test this hypothesis, the effects of combinations of several inhibitors on antigen presentation in vaccinia virus-infected cells were tested.

Because most class I epitopes are generated by proteasome activity, the presentation of these epitopes was analyzed in the presence of lactacystin, together with each other inhibitor in turn. Figure 8B shows that the combination of lactacystin with $(z\text{-}LL)_2$ ketone, LEU, or PEPST did not block presentation in infected cells. In contrast, a partial blocking of presentation was observed in target cells treated with lactacystin and PHE (Fig. 8B). These results demonstrate that the $A10L_{688-696}$ and $A17L_{9-17}$ peptides were processed by both metalloproteases and proteasomes *in vivo*. Additionally, the antigen presentation of both epitopes was partially inhibited by the combination of lactacystin and LeuSH, indicating that ERAAP or other metalloaminopeptidases were involved in the generation of these viral epitopes. Metallopeptidases can be divided into aminopeptidases, endopeptidases, carboxypeptidases, and carboxydipeptidases, among other peptidase categories, based on their cleavage mechanisms (reviewed in reference 55). Some of these groups can be distinguished by the use of different specific inhibitors (summarized in Table 1). To more precisely identify the metallopeptidase group involved in both $A10L_{688-696}$ and $A17L_{9-17}$ antigen processing, target cells were infected with VACV and treated with a mixture of lactacystin and different specific inhibitors (Table 1). The caspase-1-specific inhibitor z-VAD.fmk was also included in view of the sensitivity of this cysteine protease to PHE. Remarkably, none of the different combinations of inhibitors tested prevented antigen presentation to specific CTLs (Fig. 8B). Because phosphoramidon is able to inhibit bacterial endopeptidases but does not block all metalloendopeptidases of mammalian origin, the most likely explanation for our results is that mammalian metalloendopeptidases that are not blocked by phosphoramidon are involved in the antigen processing of these viral epitopes. Over 100 different well-characterized metalloendoproteases of higher vertebrates are resistant to this drug (7); therefore, positive identification of the peptidase involved the processing of TAP-independent vaccinia epitopes awaits further characterization.

To demonstrate that two different types of metalloproteases, aminopeptidases and endopeptidases, were independently involved in the generation of the $A10L_{688-696}$ and $A17L_{9-17}$ epitopes, their presentation was analyzed in the presence of a mixture of PHE and LeuSH. The incomplete blocking detected in the presentation indicated that aminometalloproteases and metalloendopeptidases are independently needed to process both the

TABLE 3 Continued

$K2L_{16-30}$ (HLA-B27, -Cw1)	$D5R_{148-157}$ (HLA-B51)	$A50R_{294-301}$ (HLA-B51)	$C11R_{101-110}$ (HLA-B51, -Cw1)	$A17L_{9-25}$ (HLA-Cw1)	$D8L_{112-119}$ (HLA-Cw1)
YRLQGFTNAGIVAYK	IAMKRTLLEL	LPFGSLGI	IPSPGIMLV	MLDDFSAGAGVLDKDL	DGLIIISI
---	---	---	---	---	---
---	---	---	---	---	---
---	---	---	------V--	---	---
---	---	---	------V--	---	---
---	---	---	------V--	---	---
---	---	---	------V--	---	--I--VA-
---	---	---	---	---	---
---	---	---	---	---	---
---	---	---	---L--V--	---	--I---A-
---	---	---	------V--	---	---
---	---	---	------V--	---	---

TABLE 4 TAP-independent ligands and epitopes and their hydrophobicity

Ligand(s) or epitope(s)	Protein	Virus	Hydrophobicity[a]	Reference(s) or source
LLWTLVVLL	LMP2	EBV	2.9	38
CLGGLLTMV	LMP2	EBV	2.1	38
8 TAP^- epitopes (mean ± SD)		EBV	2.5 ± 0.2	38
MLDDFSAGA	$A17L_{9-17}$	Vaccinia virus	0.4	This study
SPEGEETII	$A10L_{614-623}$	Vaccinia virus	−0.6	This study
ILDRIITNA	$A10L_{688-696}$	Vaccinia virus	0.8	This study
SRGYFEHMKK	$A10L_{867-876}$	Vaccinia virus	−1.7	This study
WQTMYTN	$B8R_{53-59}$	Vaccinia virus	−1.2	This study
YRLQGFTNAGIVAYK	$K2L_{16-30}$	Vaccinia virus	−0.1	This study
IAMKRTLLEL	$D5R_{148-157}$	Vaccinia virus	0.7	This study
LPFGSLGI	$A50R_{294-301}$	Vaccinia virus	1.5	This study
IPSPGIMLV	$C11R_{101-110}$	Vaccinia virus	1.6	This study
MLDDFSAGAGVLDKDL	$A17L_{9-25}$	Vaccinia virus	0.3	This study
DGLIIISI	$D8L_{112-119}$	Vaccinia virus	2.1	This study
11 TAP^- ligands (mean ± SD) ($P < 0.0001$)		Vaccinia virus	0.3 ± 1.2	This study
79 TAP^+ epitopes (mean ± SD)		Vaccinia virus	0.4 ± 1.0	26, 46

[a] Values correspond to the grand average of hydropathicity (GRAVY) scale (ProtParam tool [ExPASy Proteomics Server; http://www.expasy.ch).

$A10L_{688-696}$ and $A17L_{9-17}$ epitopes (Fig. 8B). Finally, the recognition of infected cells by specific CTL was abrogated in the presence of three inhibitors: lactacystin, PHE, and LeuSH (Fig. 8B). To exclude the possibility that the inhibitory effect of lactacystin, PHE, or LeuSH was due to toxic effects on target cells or on recombinant vaccinia virus (rVV) replication rather than to a specific blocking of the respective proteases, experiments similar to those shown in Fig. 8B were performed in parallel using the same target cells and VACV-$OVA_{257-264}$, which codes for the miniprotein MSIINFEKL. Specific recognition by SIINFEKL-specific CTLs of target cells infected with the VACV-$OVA_{257-264}$ virus (64% ± 11% of $CD8^+$ cells secreted IFN-γ) was detected. In contrast, VACV-$OVA_{257-264}$-infected target cells incubated with all combinations of these three inhibitors were efficiently recognized by SIINFEKL-specific CTL and no inhibition was detected (hatched bars in Fig. 8B). These data indicate that inhibition of the $A10L_{688-696}$ and $A17L_{9-17}$ epitopes by addition of lactacystin, PHE, and LeuSH drugs is formally due to specific blockage of the respective proteases and not to blocking of rVV replication or other toxic effects.

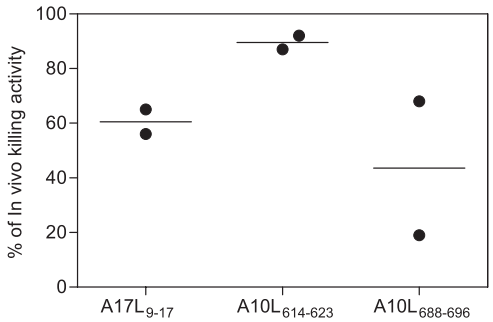


FIG 6 *In vivo* killing activity of VACV peptide-specific CTLs in HLA-A*0201-transgenic mice. HLA-A*0201-transgenic mice were infected i.p. with VAVC. One week later, they were injected i.p. with syngeneic splenocytes that were either labeled with a low level of CFSE and not subjected to pulsing or pulsed with the indicated VACV peptides and labeled with a high level of CFSE. Two days later, the remaining CFSE-labeled target cells in the peritoneal cavity were lavaged and analyzed by flow cytometry. Percent *in vivo* lysis values for individual animals (circles) ± SD (lines) are shown.

In summary, the proteasomes, aminometalloproteases, and metalloendopeptidases are all independently involved in the antigen processing of both $A10L_{688-696}$ and $A17L_{9-17}$ epitopes. The existence of identical neighboring residues around both the $A10L_{688-696}$ and $A17L_{9-17}$ sequences (Fig. 8C) could explain the similarity of the antigen-processing pathways identified in these two different HLA-restricted epitopes.

DISCUSSION

The results reported here show an exceptional diversity of TAP-independent ligands, with 11 ligands simultaneously processed and presented as part of 13 different HLA-peptide complexes in VACV-infected cells. All three of the identified HLA-A2 ligands generated long-term CTL memory responses to vaccinia virus in a transgenic mouse model. Proteasomal and nonproteasomal pathways were involved in the processing and presentation of two vaccinia virus-encoded HLA-A2-restricted antigens.

Identification of viral HLA ligands by mass spectrometry analysis contributes to a better understanding of the cellular antiviral immune response. However, the mass spectrometry strategies designed to identify such ligands have not become routine because of the difficulty in selecting and identifying the very limited number of viral sequences among the large number of self-peptides bound to HLA class I molecules. Therefore, the number of studies on viral HLA ligands is still limited. To date, only two studies have identified vaccinia virus ligands by mass spectrometry (26, 46). Here, we identified three to four TAP-independent ligands that were processed and presented by each of the four class I molecules expressed in infected cells. Neither HLA-specific elution nor CTL responses have previously been reported in TAP^+ cells for 10 of the ligands identified in our study. However, as all three TAP-independent HLA-A*0201 ligands also generated responses in TAP^+ HLA-A*0201-transgenic mice, these epitopes must also be generated in wild-type cells.

We detected HLA ligands from viral gene products expressed in the three gene expression temporality clusters of the viral life cycle (see Table S1 in the supplemental material), namely, the early (A50R, B8R, C11R, and D5R), early/late (K2L), and late (A10L, A17L, and D8L), in agreement with a previous study investigating the CTL response in the same virus strain (53). In

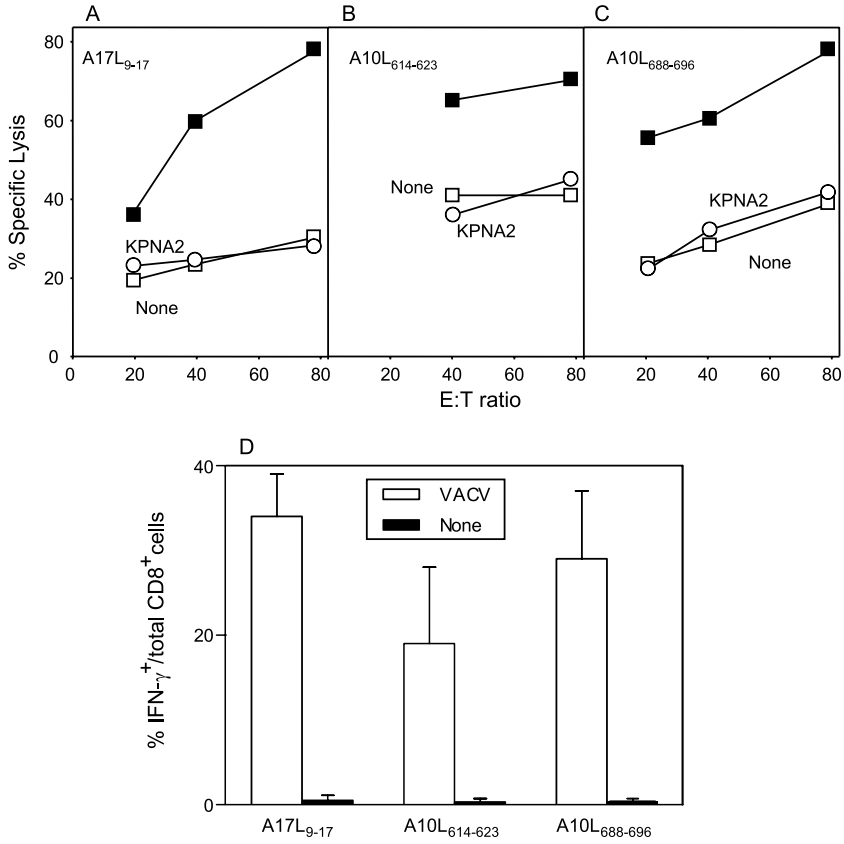


FIG 7 Recognition by CTLs of VACV epitopes presented by the HLA-A*0201 class I molecule. (A to C) RMA-HLA-A*0201 target cells prepulsed with 10^{-5} M $A17L_{9-17}$ (A), $A10L_{614-623}$ (B), and $A10L_{688-696}$ (C) synthetic peptides (filled squares) were tested in a cytolytic assay with their respective memory peptide-specific CTL lines obtained from HLA-A*0201-transgenic mice immunized up to 30 days before with VACV (upper panels). Negative controls used were unpulsed cells (open squares) and pulsed cells with the KPNA2 peptide (open circles). The data represent the means of the results of at least three independent experiments. (D) HLA-A*0201 TAP$^+$ cells were infected with VACV (open bars) or not (filled bars) at a multiplicity of infection of 40 PFU/cell and analyzed by ICS for CD8$^+$ T cell activation with the HLA-A*0201 memory peptide-specific CTL lines. The data represent the means of the results of 4 to 6 independent experiments.

similarity to the TAP$^+$ response, the TAP-independent response also sampled proteins from the entire viral life cycle of the VACV WR strain. Other authors reported the absence of presentation of peptides derived from late viral Ags to specific T cells by infected mouse target cells (29, 46), but a different VACV strain was used in their studies. The sequences we identified were derived from eight different vaccinia virus proteins (see Table S1 in the supplemental material). Five of the proteins present signal sequence or transmembrane domains in their respective amino acid sequences and are therefore accessible to HLA-containing compartments where they could be processed by resident proteases. In contrast, for three vaccinia virus proteins, A10L, A50R, and D5R, no obvious TAP-independent antigen presentation could be predicted. However, vaccinia virus DNA replication is associated with the cytoplasmic side of the rough ER, and when the replication proceeds, ER membranes are recruited (reviewed in reference 60). Late in infection, the ER envelope is disassembled, and these membranes are recycled to the ER (reviewed in reference 60). During this complex process, which includes deep membrane reorganization, some molecules of A10L, A50R, and D5R proteins could gain access to a TAP-independent antigen-processing pathway.

In most cases, the identified natural MHC class I ligands had the canonical anchor motifs, and their respective antigenicity and MHC class I binding affinity data were correlated. This suggests that only high-affinity peptides are recognized by CTLs and that epitopes of low affinity may be immunologically irrelevant. We have demonstrated that cytotoxic responses are targeted against both high- and low-affinity HLA-A*0201 ligands of VACV; therefore, the TCRs of individual CTLs specific for low-affinity epitopes must display a compensatory high affinity. In addition, it has been reported that low-affinity self-peptides in autoimmunity (reviewed in reference 16) or tumor peptides (4) generate specific responses. These findings suggest that the range of peptides that can generate CTLs is broader than was formerly thought and that the role of low-affinity epitopes in antiviral responses must be evaluated in future studies.

Different MHC class I alleles have different TAP dependencies. The widespread HLA-A2 allele is considered to be the least TAP dependent (63). Alleles such as HLA-B7 and HLA-B8 can also bind ligands that are dependent on mechanisms other than TAP transport, while other MHC class I molecules, including HLA-A3, HLA-A24, and HLA-B27, have been described as mainly TAP dependent (2). In the present report, several TAP-independent ligands were identified for alleles with different TAP requirements. Thus, the overall expression of MHC class I molecules with endogenous bound peptides is not indicative of specific TAP-independent cellular responses, which must be studied individually for each specific virus.

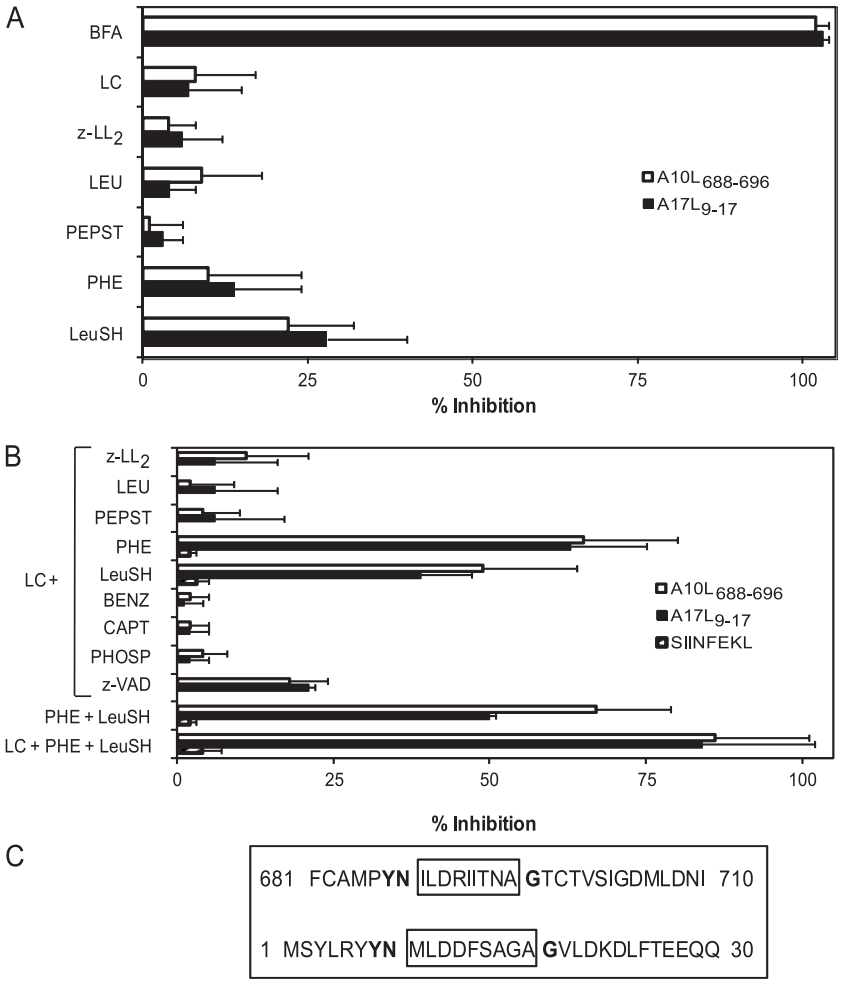


FIG 8 Recognition of target cells infected in the presence of inhibitors. (A and B) RMA-HLA-A*0201 target cells infected for 16 h with VACV or VACV-$OVA_{257-264}$ at a multiplicity of infection of 40 PFU/cell were treated with the indicated inhibitors. An ICS assay was used to test for recognition by $A10L_{688-696}$-specific (open bars), $A17L_{9-17}$-specific (closed bars), or SIINFEKL-specific (hatched bars) CTLs. The percentages of specific inhibition obtained by the addition of the indicated single inhibitor (A) or mixture of two or three inhibitors (B) were calculated as follows:

$$\%\ \text{specific inhibition} = 100 - \frac{[(\text{ICS VAVC} + \text{inhibitor}) - \text{ICS without infection}]}{\text{ICS VACV} - \text{ICS without infection}} \times 100$$

where the data displayed represent the means ± SD of the results of 4 to 6 independent experiments. (C) The sequences of the residues neighboring the epitopes in A10L and A17L proteins. The sequence of each epitope is boxed. Residues that are identical in the two sequences are indicated with boldface characters. LC, lactacystin.

Although TAP-independent viral epitopes are known (reviewed in references 13, 27, and 36), only one epitope has been identified as the target of a specific antiviral CTL response in most of the cases studied. No systematic studies of TAP-independent pathways with a single virus and different HLA molecules have been reported. The exception is EBV, for which CTLs from different donors recognize several viral epitopes from two different viral proteins restricted by several HLA class I molecules in TAP-negative cell backgrounds (reviewed in reference 38). Here, we report a second case with 11 ligands from eight different viral proteins presented by four different HLA class I molecules in the same TAP-deficient vaccinia virus-infected cells. The simultaneous presentation of the elements of this broad complexity of viral peptide-MHC complexes can help to explain why TAP-deficient patients do not seem particularly susceptible to viral infections and may appear asymptomatic for long periods of their lives. In addition, the existence of multiple TAP-independent ligands in two very different viruses, a gammaherpesvirus and an orthopoxvirus, suggests that these pathways could represent an extended but perhaps secondary mechanism that forms part of the multiple layers of defense against viral infection and that the significance of these alternative processing pathways *in vivo* needs to be further studied.

The involvement of the following proteases in the processing of endogenously synthesized antigens in a manner independent of that seen with the classical proteasome pathway has been reported previously (reviewed in reference 12). In the present study, two HLA-A2-restricted epitopes were processed in parallel by amino-metallopeptidases as well as by metalloendoproteases and a classic proteasomal pathway.

In addition to the $A17L_{9-17}$ epitope previously described in a study performed using TAP-sufficient cells (5), two distinct peptide epitopes recognized by human HLA-A2-restricted $CD8^+$ T

TABLE 5 Comparison between predicted and experimentally detected binding of vaccinia virus ligands to HLA molecules

Ligand	Sequence	HLA	
		Predicted[a]	Experimentally detected
$A17L_{9-17}$	MLDDFSAGA	A2	A2
$A10L_{614-623}$	SPEGEETII	B51	A2
$A10L_{688-696}$	ILDRIITNA	A2	A2
$A10L_{867-876}$	SRGYFEHMKK	B27	B27
$B8R_{53-59}$	WQTMYTN	B27	B27
$K2L_{16-30}$	YRLQGFTNAGIVAYK	B27	B27/Cw1
$D5R_{148-157}$	IAMKRTLLEL	B51/Cw1	B51
$A50R_{294-301}$	LPFGSLGI	B51	B51
$C11R_{101-110}$	IPSPGIMLV	B51	B51/Cw1
$A17L_{9-25}$	MLDDFSAGAGVLDKDL	A2/Cw1	Cw1
$D8L_{112-119}$	DGLIIISI	B51	Cw1

[a] Data are from the SYFPEITHI (http://www.syfpeithi.de), BIMAS (http://www-bimas.cit.nih.gov), and IEDB (http://www.immuneepitope.org) databases.

cells and eight HLA-B27, HLA-B51, and/or HLA-Cw1 ligands were newly detected in this study. Nine of the viral ligands identified are conserved in all three vaccinia virus strains, seven orthopoxviruses (including cowpox virus), and two variola strains. In contrast, only 60% of the previously described vaccinia virus epitopes in the variola virus proteome are identical (46, 50, 53). Therefore, TAP-independent vaccinia ligands are more highly conserved between immunogenic and pathological poxviruses than TAP-dependent epitopes and could be more specific targets for immunization. Cowpox virus, the first component of early vaccines, specifically inhibits TAP-dependent peptide translocation (1); therefore, TAP-independent epitopes conserved between variola virus and cowpox virus are probably responsible for the initial cross-protection. Additionally, truncated gene fragments similar to those encoding the TAP-blocking CPXV12 protein are widely found in poxvirus genomes (Poxvirus Bioinformatics Resources Center; http://www.poxvirus.org) and frequently represent loss-of-function phenotypes, but this "genetic debris" may acquire entirely new, unanticipated functions. Therefore, the conservation of epitopes between different vaccine strains and pathogens is relevant for vaccine design and suggests that ligands from TAP-independent pathways could be interesting candidates for inclusion in immunization protocols. This idea is also important with respect to bioterrorism, because some countries did not participate in the WHO smallpox eradication program and, at this time, no information about the elimination of their samples of the pandemic virus has been provided.

Computational methods designed for predicting MHC-peptide binding are increasingly being used to identify epitopes for vaccine design and to monitor T cell responses (reviewed in references 35 and 44). The prediction of MHC-peptide binding is far from perfect, as our results indicate. The prediction by bioinformatics tools of HLA class I molecules presenting the nine vaccinia virus ligands identified in the current study showed several different mistakes, including incomplete HLA identifications ($K2L_{16-30}$ and $C11R_{101-110}$ ligands), ambiguous HLA restrictions ($D5R_{148-157}$ and $A17L_{9-25}$ ligands), and wrong assignations ($A10L_{614-623}$ and $D8L_{112-119}$ ligands) (Table 5). In summary, more than half of these ligands showed inconsistencies between the computational predictions and experimentally detected HLA restriction. These results reveal the limitations of predictive methods for identifying natural MHC class I ligands and T cell epitopes. The current analytical algorithms may not be sufficiently accurate and should be used with caution.

Collectively, the results in the current report highlight the importance of analyzing natural peptides that result from the endogenous processing of viral proteins and demonstrate the complexity and plasticity of MHC-peptide interactions. This analysis is of fundamental importance for gaining a detailed understanding of MHC class I-restricted immunity and for future vaccine design.

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SUPPLEMENTAL FIGURE LEGENDS

Figure S1. VACV infection of the RMA-HLA-A*0201 cell line in presence of different protease inhibitors.

RMA-HLA-A*0201 target cells infected for 16 hr with VACV at a multiplicity of infection of 40 plaque-forming units/cell were treated with the indicated inhibitors. A mock infected control was included as negative control. The cells were stained with the Omnitope antiserum-FITC that recognizes VACV purified virions. Samples were analyzed by FACS. The results, calculated as fluorescence index ± SD, are the mean of 4 independent experiments. The fluorescence index was calculated as the ratio of mean channel fluorescence of the sample to that of the control incubated without VACV. All VACV-infected conditions (with and without inhibitors) show significant P values ($P < 0.01$) versus mock infected controls. In contrast, all inhibitor conditions show non significant P values versus VACV-infected control without an inhibitor.

Figure S2. Identification of three HLA-A*0201 ligands in infected cell extracts by mass spectrometry.

MS/MS fragmentation spectra obtained from quadrupole ion trap mass spectrometry of the ion peaks at *m/z* 926.4 (upper left panel), *m/z* 974.6 (medium left panel), and *m/z* 514.8 (lower left panel) from the VACV-infected cell extract and the corresponding synthetic peptide (right panels). The vertical axis represents the relative abundance of the parental ion and each fragmentation ion detected. The horizontal axis corresponds to the m/z region in which significant daughter ions were detected. Ions generated by the fragmentation are detailed, and the sequence deduced from the indicated fragments is shown in the upper left box of each panel.

Figure S3. Identification of three HLA-B27 ligands in infected cell extracts by mass spectrometry.

MS/MS fragmentation spectra obtained from quadrupole ion trap mass spectrometry of the ion peaks at *m/z* 428.2 (upper left panel), *m/z* 567.6 (medium left panel), and *m/z* 472.1 (lower left panel) from the VACV-infected cell extract and the corresponding synthetic peptide (right panels). The axes are as described in Figure S1.

Figure S4. Identification of three HLA ligands in infected cell extracts by mass spectrometry (I).

MS/MS fragmentation spectra obtained from quadrupole ion trap mass spectrometry of the ion peaks at *m/z* 594.4 (upper left panel), *m/z* 803.5 (medium left panel), and *m/z* 926.5 (lower left panel) from the VACV-infected cell extract and the corresponding synthetic peptide (right panels). The axes are as described in Figure S1.

Figure S5. Identification of three HLA ligands in infected cell extracts by mass spectrometry (II).

MS/MS fragmentation spectra obtained from quadrupole ion trap mass spectrometry of the ion peaks at *m/z* 843.5 (upper left panel), *m/z* 833.9 (medium left panel), and *m/z* 851.0 (lower left panel) from the VACV-infected cell extract and the corresponding synthetic peptide (right panels). The axes are as described in Figure S1.

Supplemental Table 1

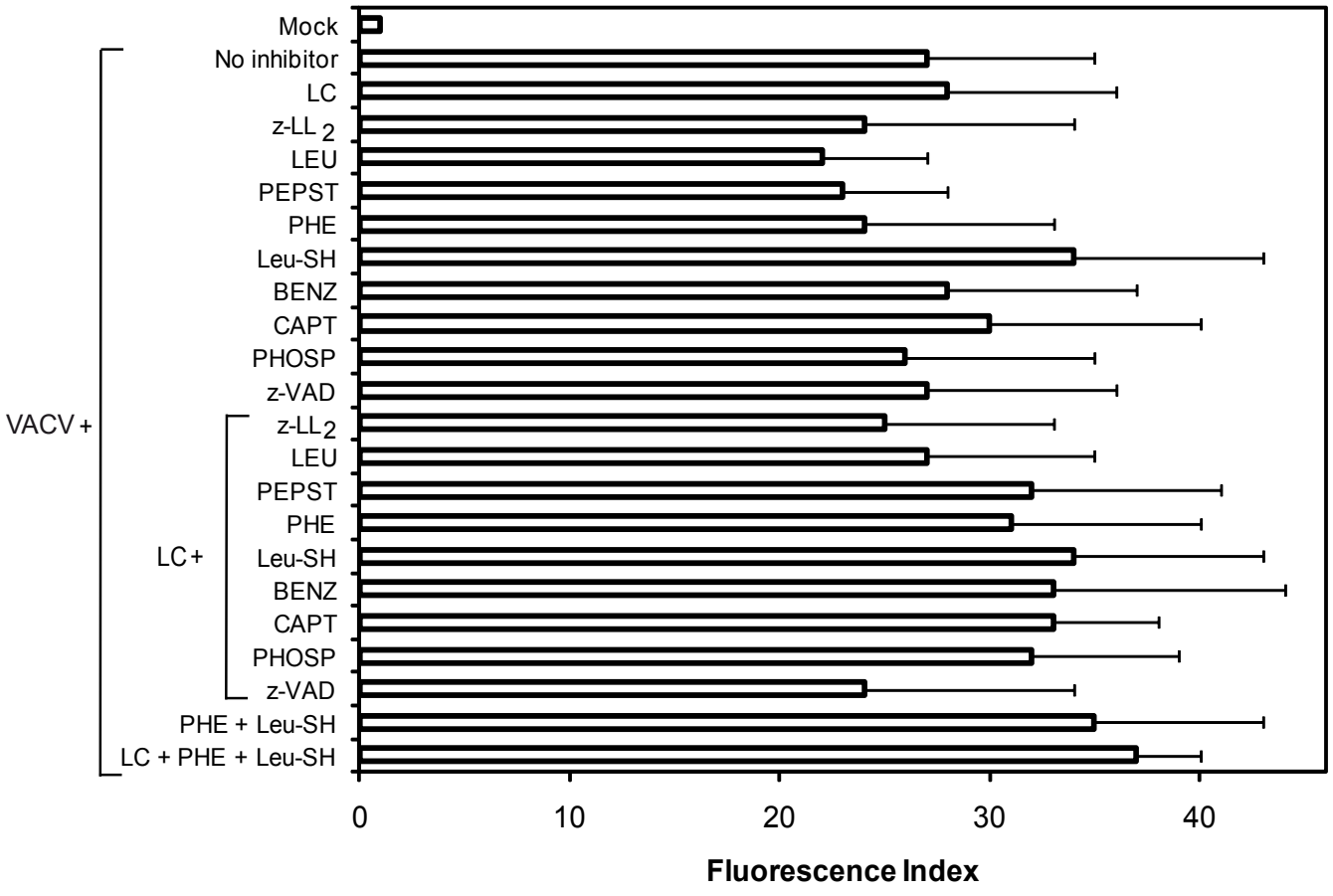
Major characteristics of HLA ligand-containing vaccinia proteins [a]

Protein	Name	Expression	Signal sequence	Transmembrane domains	Encapsidated	pI	Mw	PTM [b]	Essential to VACV
A10L	P4a precursor	Late	None	0	Yes, in core	6.1	102		NI [c]
A17L	IMV membrane protein	Late	Putative	4	Yes, in membrane	4.4	23	Phosphorylation	Yes
A50R	DNA ligase	Early	None	0	No	7.9	63		No
B8R	IFN-gamma receptor	Early	Putative	0	No	5.5	31		NI
C11R	EGF growth factor	Early	Putative	1	No	6.4	16		NI
D5R	NTPase	Early	None	0	No	6.8	90		Yes
D8L	Carbonic anhydrase	Late	None	1	Yes, in membrane	8.8	35		NI
K2L	Serpin 1,2,3	Early/Late	Putative	0	No	9.0	42		NI

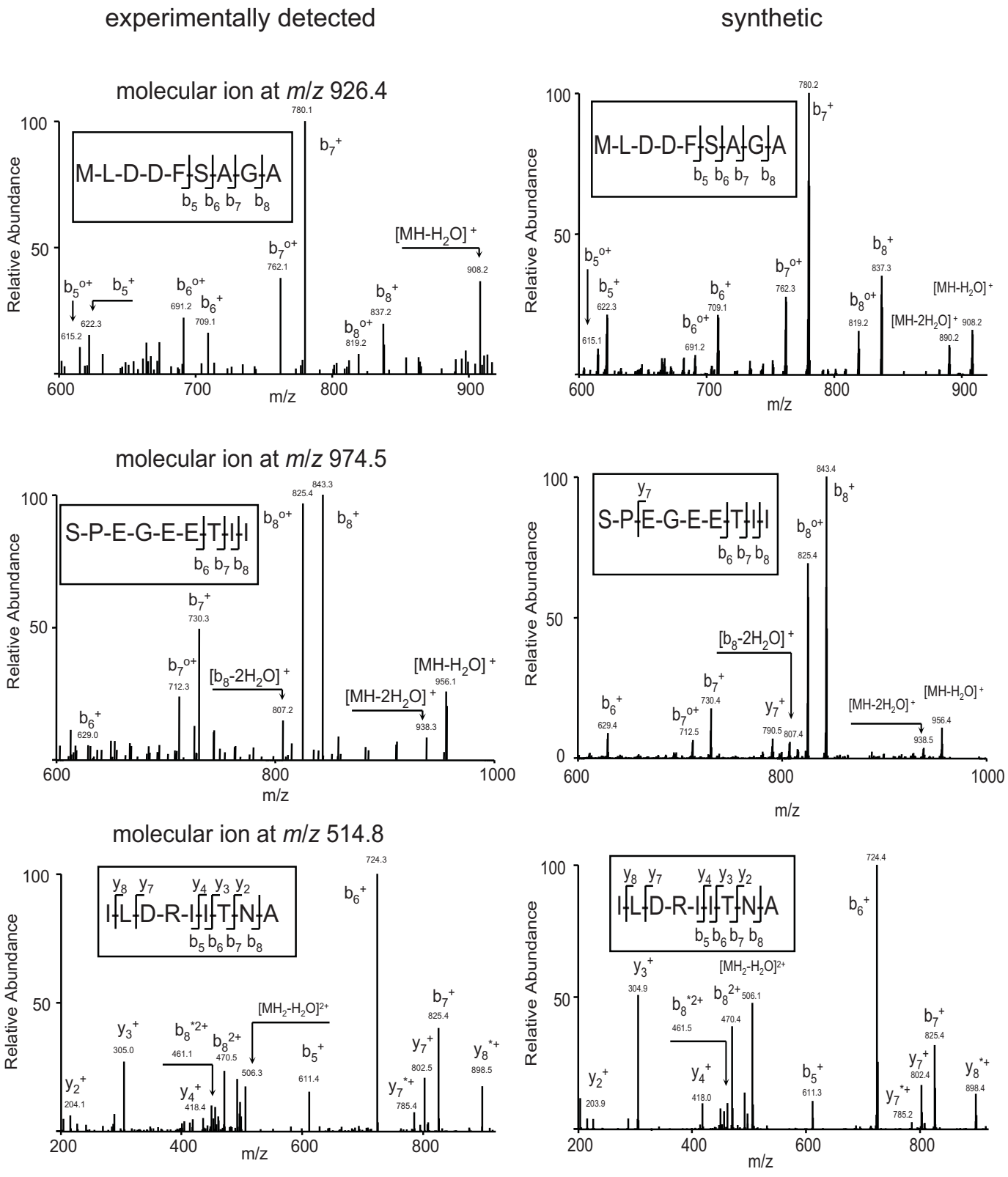
[a] All information was obtained from the Poxvirus Bioinformatics Resources Center (http://www.poxvirus.org).

[b] PTM, post-translational modification.

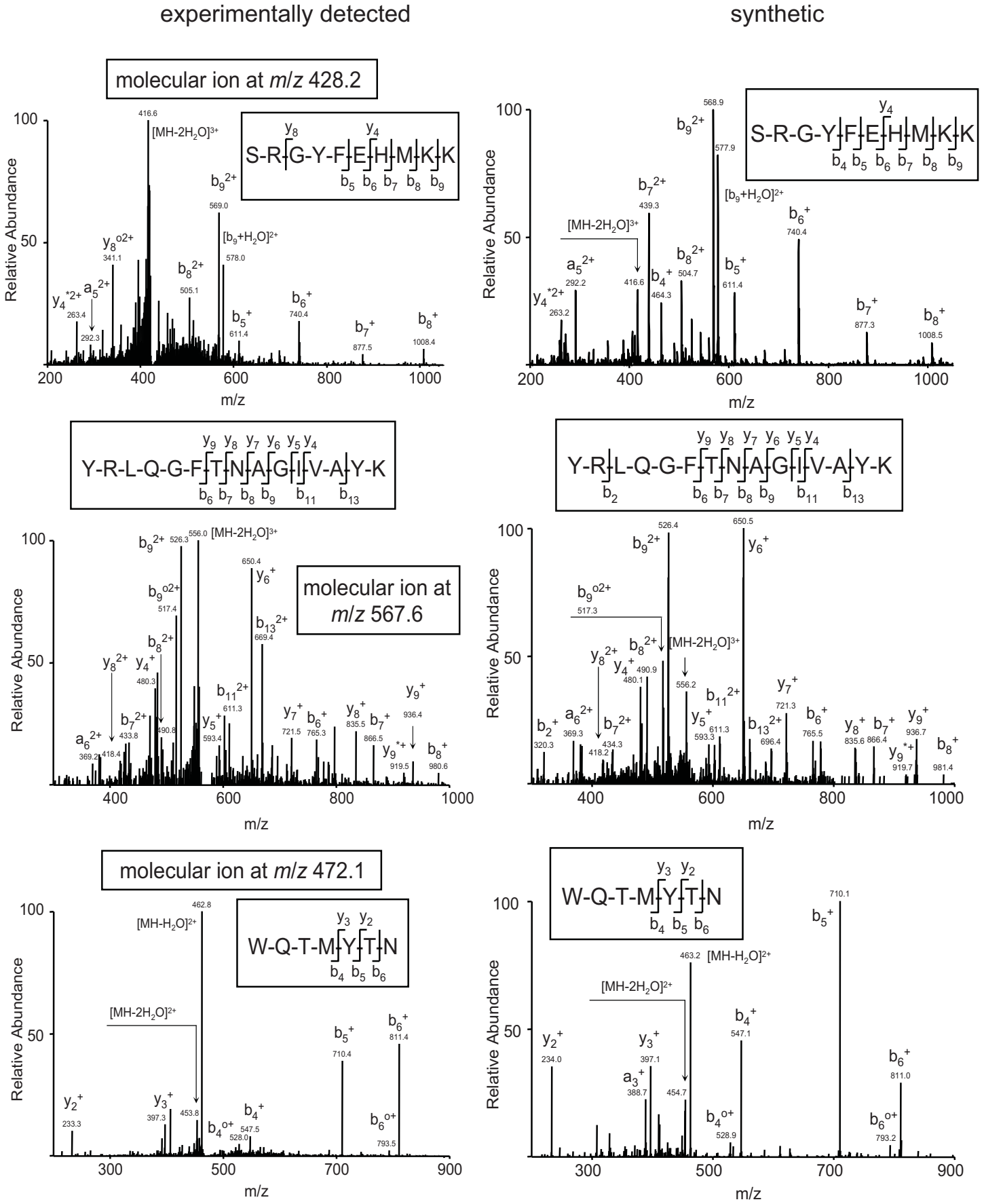
[b] NI, no information.



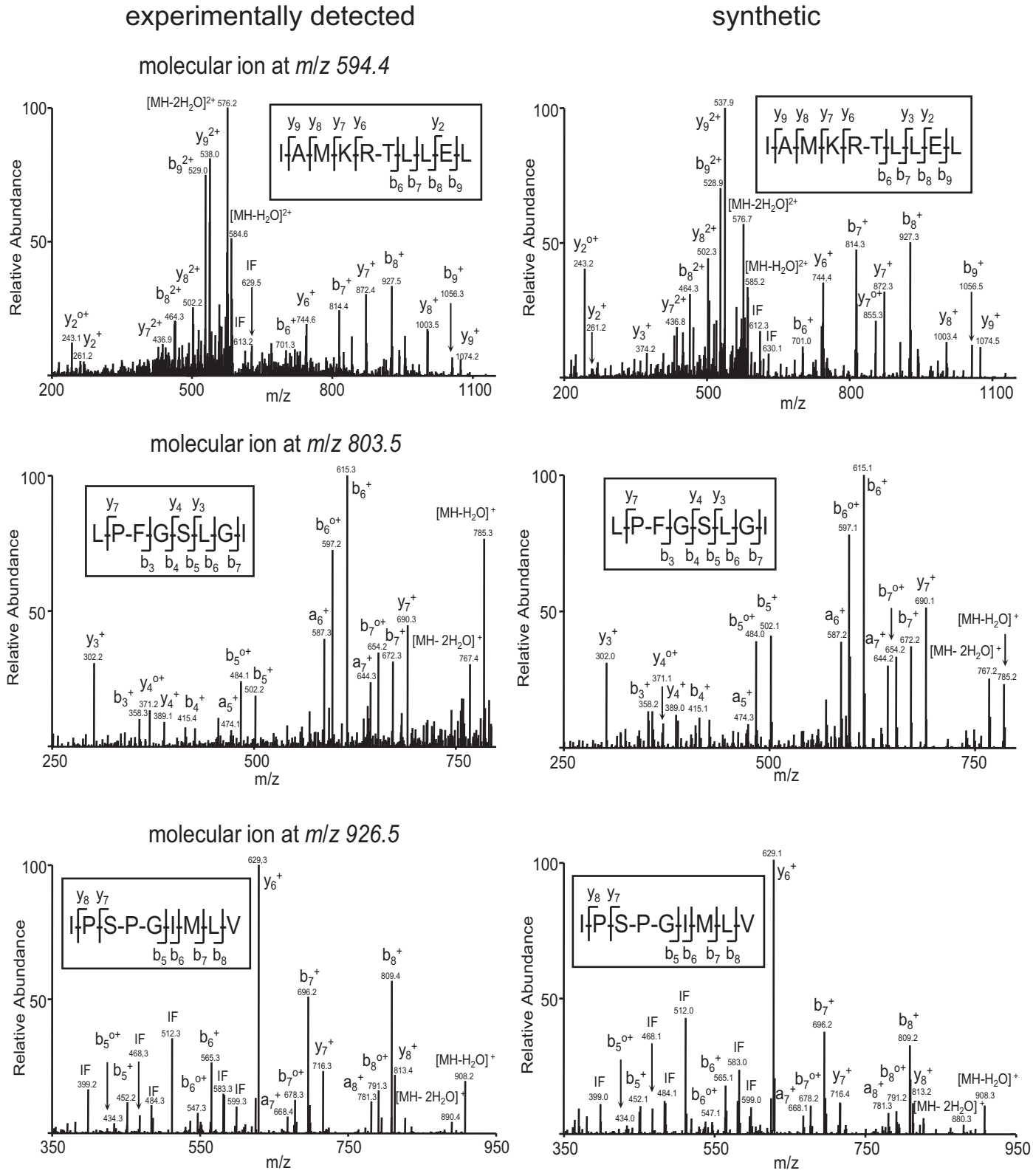
Lorente et al. Figure S1



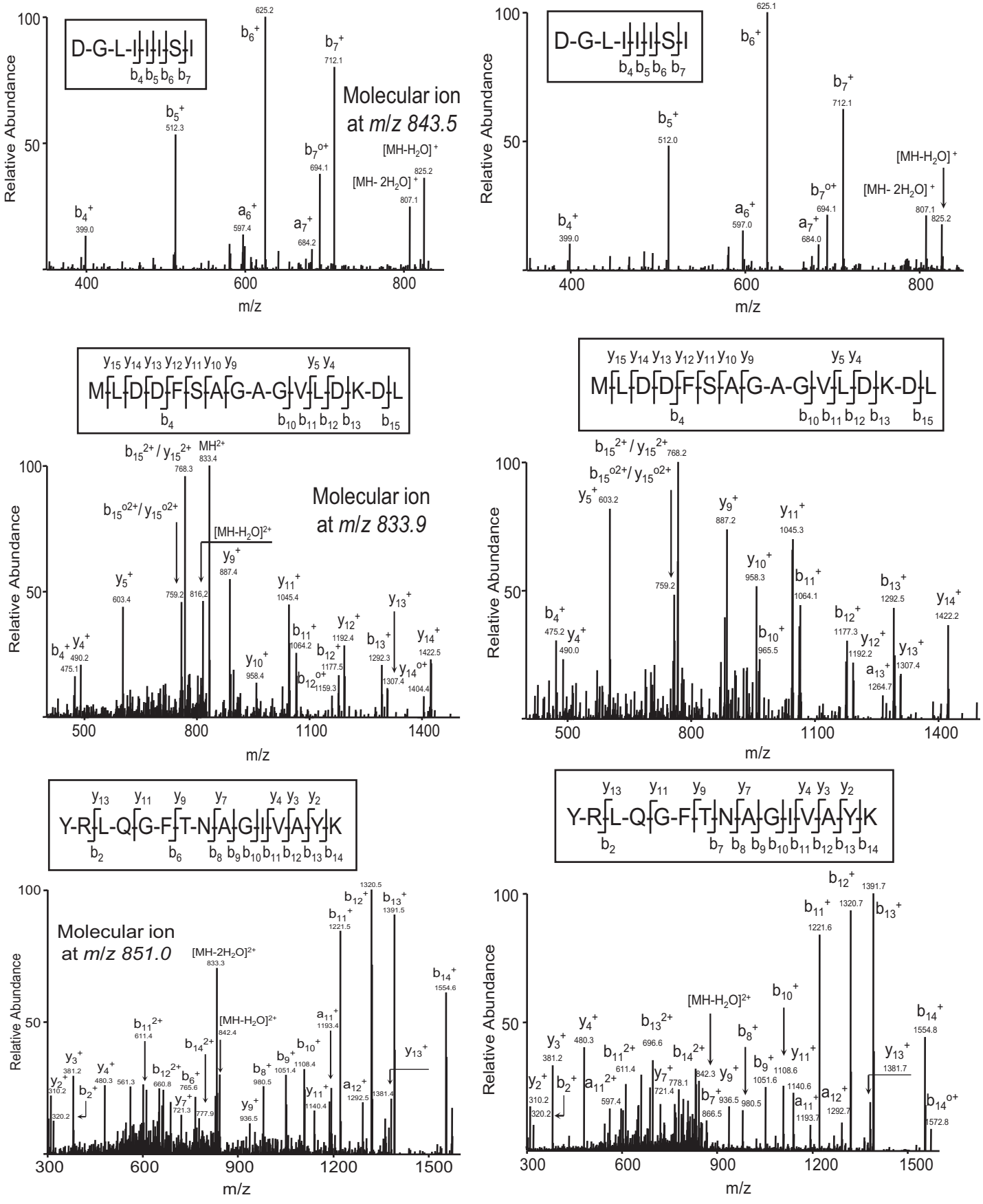
Lorente et al. Figure S2



Lorente et al. Figure S3



Lorente et al. Figure S4



Lorente et al. Figure S5

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A Viral, Transporter Associated with Antigen Processing (TAP)-independent, High Affinity Ligand with Alternative Interactions Endogenously Presented by the Nonclassical Human Leukocyte Antigen E Class I Molecule*

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Background: Individuals with nonfunctional transporter associated with antigen processing (TAP) present ligands generated by TAP-independent processing pathways associated with classical HLA class I molecules.
Results: A vaccinia virus ligand is efficiently presented by nonclassical HLA-E using alternative interactions.
Conclusion: Nonclassical HLA-E presents viral ligands.
Significance: This expands the role of HLA-E as an antigen-presenting molecule.

The transporter associated with antigen processing (TAP) enables the flow of viral peptides generated in the cytosol by the proteasome and other proteases to the endoplasmic reticulum, where they complex with nascent human leukocyte antigen (HLA) class I. Later, these peptide-HLA class I complexes can be recognized by CD8$^+$ lymphocytes. Cancerous cells and infected cells in which TAP is blocked, as well as individuals with unusable TAP complexes, are able to present peptides on HLA class I by generating them through TAP-independent processing pathways. Here, we identify a physiologically processed HLA-E ligand derived from the D8L protein in TAP-deficient vaccinia virus-infected cells. This natural high affinity HLA-E class I ligand uses alternative interactions to the anchor motifs previously described to be presented on nonclassical HLA class I molecules. This octameric peptide was also presented on HLA-Cw1 with similar binding affinity on both classical and nonclassical class I molecules. In addition, this viral peptide inhibits HLA-E-mediated cytolysis by natural killer cells. Comparison between the amino acid sequences of the presenting HLA-E and HLA-Cw1 alleles revealed a shared structural motif in both HLA class molecules, which could be related to their observed similar cross-reactivity affinities. This motif consists of several residues located on the floor of the peptide-binding site. These data expand the role of HLA-E as an antigen-presenting molecule.

CD8$^+$ cytolytic T lymphocyte-mediated recognition and killing of virally infected cells first requires proteolytic degradation of viral proteins by the proteasome and other cytosolic proteases (1). This degradation generates short peptides of 8–11 amino acids, which are then translocated to the endoplasmic reticulum lumen by transporter associated with antigen processing (TAP),[2] where they assemble with newly synthesized HLA class I heavy chain and β2-microglobulin.

Humans and mice with mutations in the TAP gene that generate nonfunctional TAP complexes have been described (2, 3). This TAP deficiency implies reduced functionality of the CD8$^+$ population, but TAP-deficient patients are not particularly susceptible to viral infections or neoplasms. Thus, TAP-independent HLA class I loading pathways may be sufficient to control these diseases and allow these individuals to live with only a increased susceptibility to chronic respiratory bacterial infections. In addition, several strains of viruses have specific mechanisms to block TAP expression or to prevent CD8$^+$ lymphocytes from identifying infected cells (reviewed in Ref. 4); therefore, the TAP-independent pathways must also be important for killing cells infected with these viruses.

Early administration of the cowpox virus, which encodes for a TAP-blocking protein (5), was the inspiration for the massive worldwide cross-protective vaccination by vaccinia virus (VACV) that eradicated pandemic smallpox, a disease caused by the variola major virus (6). The *Orthopoxvirus* vaccinia is a widely used tool for research and vaccine development (7). Currently, bioterrorism and emerging infectious diseases have elicited renewed interest in VACV and other poxviruses (8). VACV administration generates a strong humoral response leading to viral clearance, and the role of cytotoxic T lymphocyte responses in this cross-protection is well documented (9, 10). During the last several years, studies in both HLA-transgenic mouse models and vaccinated humans have identified more

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[2] The abbreviations used are: TAP, transporter associated with antigen processing; HLA, human leukocyte antigen; VACV, vaccinia virus; Ab, antibody.

than 70 VACV-derived epitopes presented by various HLA molecules (11, 12).

HLA-E is a nonclassical class I molecule that binds monomorphic signal peptides derived from classical HLA class I proteins. This complex is the ligand of innate receptors expressed mainly by natural killer cells and thereby regulates lymphocyte activity (13). Several recent studies have indicated that HLA-E complexed with pathogen-derived peptides could be recognized by $CD8^+$ T cells (14). In addition, binding to HLA-E has been demonstrated for some viral peptides that were previously thought to bind the classical HLA-A2 class I molecule (15, 16).

In a previous study using mass spectrometry to analyze HLA-bound peptide pools isolated from large numbers of TAP-deficient VACV-infected cells, we identified eleven ligands that were naturally presented by four different HLA-A, -B, and -C class I molecules (17). Of these, six were obtained by immunoprecipitation with the mAb W6/32, which is specific for a monomorphic HLA class I determinant (18). Later, to identify the HLA restriction of these ligands, HLA-peptide complex stability assays were performed using the TAP-deficient T2 cells with specific anti-HLA mAbs (17). Two of these VACV ligands were endogenously presented by HLA-B51 in human TAP-deficient cells, and another three were presented by HLA-Cw1 class I molecules. In addition, one VACV ligand, $C11R_{101-110}$, was presented by both classical HLA-B51 and -Cw1 class I molecules in infected cells. Because the mAb W6/32 used in the HLA immunoprecipitation recognizes a conformational epitope on human HLA class I molecules, including the nonclassical HLA-E allele (19), some of these six VACV ligands could also have additional binding ability and could be presented by the HLA-E allele in T2 TAP-deficient VACV-infected cells. In the present study, we explore a possible role for HLA-E in presenting some of the previously described TAP-independent VACV ligands.

EXPERIMENTAL PROCEDURES

Cell Lines—T2 cells are a line of TAP-deficient human cells that express HLA-A2, -B51, and -Cw1 class I molecules on their surface (20). The 721.221 cells are a HLA-A, -B, and -C null human line that express HLA-E on their surface (21). Both cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 5 μM β-mercaptoethanol. The NK3.3 natural killer cells were cultured in α-minimal essential medium supplemented with 100 units/ml recombinant human IL-2 and 25% FBS (22). Recombinant human IL-2 was generously provided by Hoffmann-La Roche for the long term propagation of NK3.3 cell line.

Synthetic Peptides—Peptides were synthesized with a peptide synthesizer (model 433A; Applied Biosystems, Foster City, CA) and were purified by reverse phase HPLC. The monosubstituted Ala analogues of VACV D8L peptide (DGLIIISI) were named according to the position of the substituted residue. Thus, A3 refers to the octamer of sequence DGAIIISI. The correct molecular mass of the peptides was established by MALDI-TOF MS, and their correct composition was determined by MS/MS on a quadrupole ion trap micro-HPLC.

HLA-Peptide Stability Assays—The following synthetic peptides were used as controls in HLA-peptide complex stability assays: KPNA2 (GLVPFLVSV, HLA-A2-restricted) (23), HBV HBc_{19-27} (LPSDFFPSV, HLA-B51-restricted) (24), CMV $pp65_{7-15}$ (RCPEMISVL, HLA-Cw1-restricted) (25), the leader peptide of HLA (VMAPRALLL, HLA-E-restricted), and C4CON (QYDDAVYLK, HLA-Cw4-restricted) (26).

The T2 line of TAP-deficient cells expresses low amounts of classical MHC class I on the cell surface. For classical HLA-A2, -B51, and -Cw1 class I stability assays, T2 cells were incubated at 26 °C for 16 h in RPMI 1640 medium supplemented with 10% heat-inactivated FBS. This allows the expression of empty MHC class I molecules that lack antigenic peptide and are only stable on the cell membrane at 26 °C and not at 37 °C. Later, the cells were washed and incubated for 2 h at 26 °C with various concentrations of peptide in the same medium. The cells were then kept at 37 °C and collected for flow cytometry after 4 h (27). This assay allows for the internalization of empty HLA class I molecules and can therefore discriminate between bound and unbound peptides.

For HLA-E stability assays, T2 cells were incubated with peptides for 6 h at 37 °C in culture medium before immunofluorescent staining as described previously (15). This treatment enhances cell surface expression of HLA-E class I molecules bearing specific HLA-E-bound peptides (15).

HLA expression levels were measured using the following Abs: monoclonal 3D12 (anti-HLA-E) (28), monoclonal PA2.1 (anti-HLA-A2) (29), polyclonal H00003106-B01P (specific for HLA-B class I molecules) (Abnova, Taipei, Taiwan), and polyclonal SC-19438 (specific for HLA-C class I molecules) (Santa Cruz Biotechnology, Santa Cruz, CA) as previously described (30). The samples were assayed on a FACSCanto flow cytometer (BD Biosciences) and analyzed using CellQuest Pro 2.0 software (BD Bioscience). The cells incubated without peptide had peak fluorescence intensities similar to the background staining observed with the secondary Ab alone or isotypic controls. The fluorescence index was calculated as the ratio of the mean channel fluorescence of the sample to that of control cells incubated without the peptides. Peptide binding was also expressed as EC_{50}, which is defined as the molar concentration of the peptides producing 50% of the maximum fluorescence obtained at a concentration range between 0.001 and 100 μM.

T Cell Line and Cytotoxicity Assays—Cytotoxicity assays were performed using the 721.221 cell line as target (T) cells and NK3.3 cell line as effector (E) cells. Before performing the assay 1×10^5 target cells were incubated overnight at 26 °C either in the absence or in the presence of the indicated peptides at 100 μM. A 2-h ^{51}Cr release assay was performed as previously described (21). Specific lysis was calculated as [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100. The spontaneous release never exceeded 20%.

Statistical Analysis—To analyze statistical significance, an unpaired Student *t* test was used. p values < 0.001 were considered to be significant.

Molecular Dynamics: Starting Structures—The native leader peptide HLA-E-binding peptide was taken from chains A, B, and C of the 3bzf Protein Data Bank file. The $D8L_{112-119}$ peptide bound to HLA-E model was built with the MODELLER9v7 program using the 3bzf Protein Data Bank file as template. The

protonation states of the ionizable groups for the three system were calculated using the H++ server (31) (32). The positions of hydrogen atoms, standard atomic charges, and radii for all the atoms were assigned according to the ff03 force field (33). The complexes were immersed in cubic boxes of TIP3P water molecules that were large enough to guarantee that the shortest distance between the solute and the edge of the box was more than 13 Å (34). Counter ions were also added to maintain electroneutrality. Three consecutive minimizations were performed: the first involved only hydrogen atoms, the second involved only the water molecules and ions, and the third involved the entire system.

Simulation Details—The initial minimized structures, prepared as stated before, were simulated in the NPT ensemble using Periodic Boundary Conditions and Particle Mesh Ewald to treat long range electrostatic interactions. The systems were then heated and equilibrated in two steps. The first step involved 200 ps of MD heating the whole system from 100 to 300 K, and the second involved equilibration of the entire system during 1.0 ns at 300 K. The equilibrated structures were the starting points for the 10-ns MD simulations at constant temperature (300 K) and pressure (1 atm). The SHAKE algorithm was used to keep bonds involving hydrogen atoms at their equilibrium length, allowing a 2-fs time step for the integration of Newton's equations of motion. ff03 and TIP3P force fields, as implemented in AMBER 10 package, were used to describe the proteins, the peptides, and the water molecules, respectively. Sample frames at 20-ps intervals from the molecular dynamics trajectory were subsequently used for analysis.

Interaction Energies Analysis—Effective binding free energies between the peptides and HLA-E were estimated using the MM-GB-SA approach as implemented in the AMBER10 package (35). The MM-GB-SA method approaches the free energy of binding as a sum of a molecular mechanics (MM) interaction term, a solvation contribution thorough a generalized Born (GB) model, and a surface area (SA) contribution to account for the nonpolar part of solvation. In addition, to better characterize peptide-protein interactions, an energy decomposition analysis in a pairwise fashion (between the peptides residues and HLA-E residues) was performed using a cutoff of 5 Å from the peptides. Polar contribution to solvation free energies were calculated with GB, whereas nonpolar were estimated to be proportional to the area lost upon binding using the linear combinations of pairwise overlaps (LCPO) method to calculate accessible surface areas (36). These calculations were performed for each snapshot from the simulations using the appropriate module within AMBER 10 package.

RESULTS

VACV D8L$_{112–119}$ Is a Noncanonical HLA-E Ligand—To explore the potential role of HLA-E as an antigen-presenting molecule of TAP-independent VACV ligands, HLA-peptide complex stability assays were performed using TAP-deficient T2 cells with an anti-HLA-E Ab. Fig. 1 shows that in contrast with a control HLA-E ligand, the leader peptide of HLA, the induction of complexes with five of the six VACV peptides tested were not detected. Thus, these viral ligands do not bind to HLA-E. In contrast, the D8L$_{112–119}$ synthetic peptide induced similar numbers of HLA-peptide surface complexes as the positive control HLA-E ligand (Fig. 1). The consensus peptide-binding motif for HLA-E is Met, Leu, or Gln at peptide position 2 (P2); Leu, Ile, Val, or Pro at P7; and Leu, Glu, or Phe C-terminal residues (37, 38). Thus, the D8L$_{112–119}$ octamer DGLIIISI is an unusual VACV ligand presented by HLA-E class I molecules.

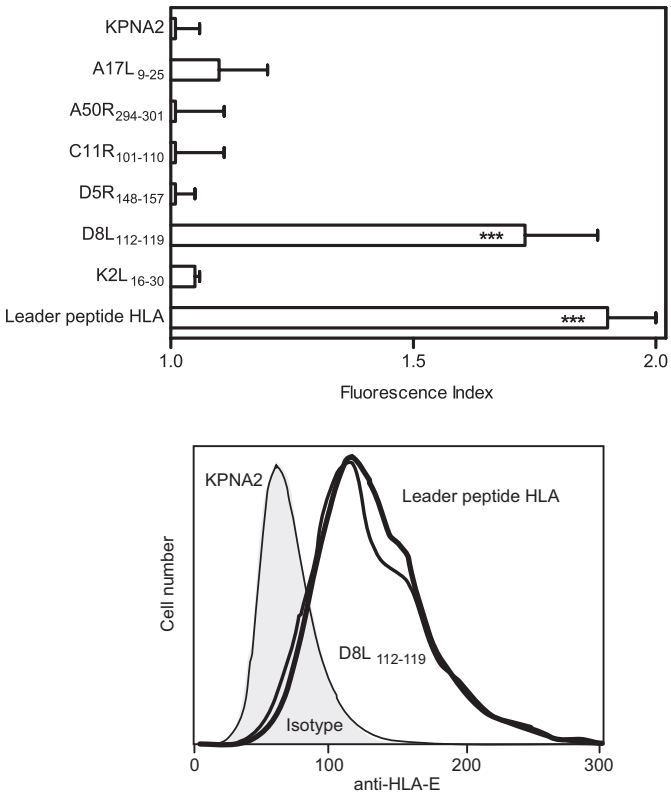
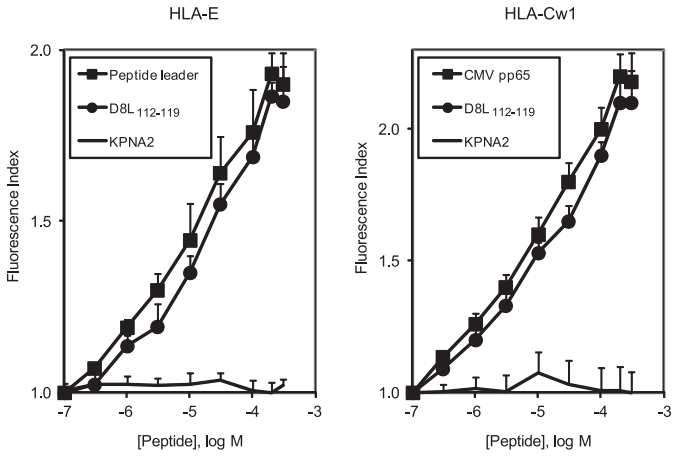


FIGURE 1. **HLA-E stabilization with synthetic VACV ligands.** The stability of HLA-E-peptide complexes on the cell surface of T2 TAP-deficient cells was measured by flow cytometry. The indicated peptides were used at 200 μM. The KPNA2 peptide and the leader peptide of HLA were used as negative and positive controls, respectively. The mAb 3D12 was used for staining. The results, calculated as fluorescence index values ± S.D., are the means of four to five independent experiments. ***, significant p values ($p < 0.001$). A representative experiment was depicted in the *bottom panel*. *Shaded histogram*, isotypic control; *thin line*, KPNA2 peptide; *medium line*, D8L 112–119; *thick line*, leader peptide of HLA.

Identical Binding Affinity to Classical HLA-Cw1 and Nonclassical HLA-E Class I Molecules for the Viral D8L$_{112–119}$ Peptide—The D8L$_{112–119}$ peptide was previously described as a HLA-Cw1-restricted ligand (17). Because the mAb 3D12 used in the current study for HLA-E binding cross-reacts with some HLA-C class I molecules, although not with HLA-Cw1 (39), HLA-peptide complex stability assays were performed using T2 cells incubated with a natural high affinity HLA-Cw1 ligand, the CMV pp65 peptide (25), and stained with the anti-HLA-E mAb 3D12 to exclude HLA-Cw1 cross-reactivity. In this case, induction of HLA complexes with the CMV pp65 peptide was not detected (data not shown). Thus, the mAb 3D12 does not bind to HLA-Cw1.

In addition, the relative affinity of D8L$_{112–119}$ to both HLA-E and -Cw1 class I molecules was evaluated. This peptide bound to HLA class I molecules in the range commonly found among natural ligands (Fig. 2). This octamer efficiently stabilized



Peptide	EC_{50} (μM)
Leader peptide	7 ± 4
D8L 112-119	14 ± 3

Peptide	EC_{50} (μM)
CMV pp65	12 ± 4
D8L 112-119	18 ± 7

FIGURE 2. **Binding affinity to HLA-E and -Cw1 of VACV D8L$_{112–119}$ synthetic peptide.** The synthetic peptide VACV D8L$_{112–119}$ (*circles*) was titrated bound to HLA-E (*left panel*) or HLA-Cw1 (*right panel*) on T2 TAP-deficient cells, and stabilization of HLA was measured by flow cytometry. The KPNA2 peptide was used as a negative control (*solid line*). The leader peptide of HLA and CMV pp65$_{7–15}$ were used as positive controls (*squares*) for binding to the HLA-E and -Cw1 alleles, respectively. The Abs used were monoclonal 3D12 (anti-HLA-E, *left panel*), and polyclonal SC-19438 (anti-HLA-C class I molecules, *right panel*). The data calculated as EC_{50} values ± S.D. are shown below and are the means of three to five independent experiments.

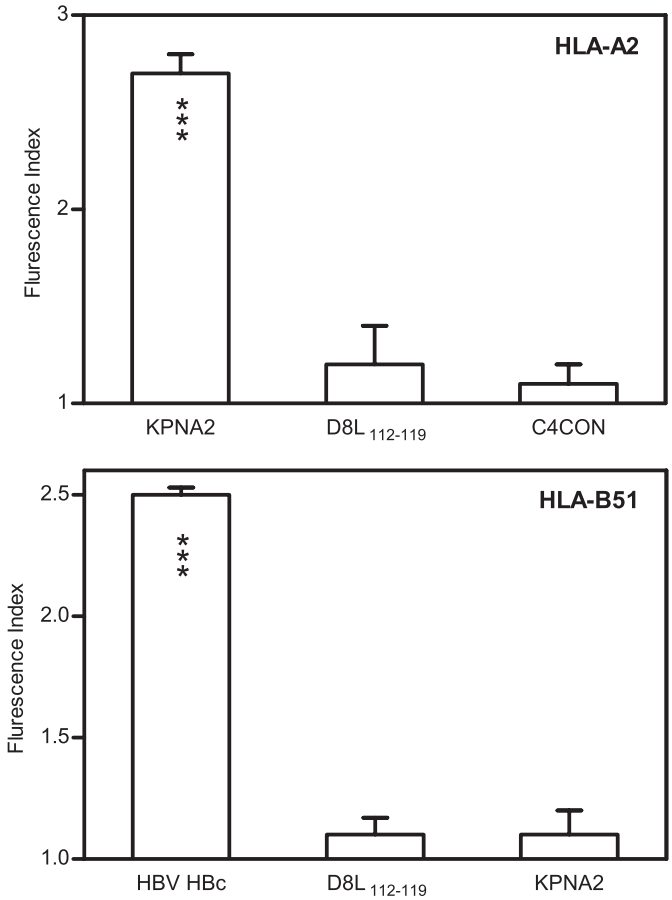


FIGURE 3. **HLA-A2 and -B51 stabilization assay with synthetic VACV D8L$_{112–119}$ ligand.** The stability of HLA-A2-peptide (*upper panel*) or HLA-B51-peptide (*lower panel*) complexes on the surface of T2 TAP-deficient cells were measured by flow cytometry. The indicated peptides were used at 200 μM. The KPNA2 and the HBV HBc peptides were used as positive controls for binding to the HLA-A2 and -B51 alleles, respectively. The C4CON and the KPNA2 peptides were used as negative controls for binding to the HLA-A2 and -B51 alleles, respectively. The Abs used were monoclonal PA2.1 (anti-HLA-A2, *upper panel*) and polyclonal H00003106-B01P (anti-HLA-B class I molecules, *lower panel*). The results, calculated as in Fig. 1, are the mean of four independent experiments. ***, significant p values ($p < 0.001$).

HLA-E (Fig. 2, *left panel*) and HLA-Cw1 (Fig. 2, *right panel*) expression on cells, with an EC_{50} for MHC binding of 14 ± 3 and 18 ± 7 μM, respectively, confirming their dual presentation. These EC_{50} values are similar to those of the other natural high affinity ligands used as positive controls. In summary, the VACV D8L$_{112–119}$ octamer is a TAP-independent high affinity ligand presented by both classical and nonclassical class I molecules.

The D8L$_{112–119}$ Peptide Is Not a HLA-A2 or -B51 Ligand—Some viral HLA-E-binding ligands were previously described as HLA-A2-restricted epitopes (15, 16). In addition, HLA-A2 and -Cw1 class I molecules present some peptides with similar anchor motifs (SYFPEITHI database (40)). Thus, HLA-peptide complex stability assays were performed to study possible HLA-A2 cross-reactivity of D8L$_{112–119}$ ligand. Fig. 3 (*upper panel*) shows that induction of HLA-A2 complexes with the vaccinia D8L$_{112–119}$ peptide was not detected, in contrast to a control HLA-A2 ligand, the KPNA2 peptide. Thus, this viral ligand does not bind to HLA-A2.

Because the HLA-B51 was also expressed in the T2 cell line, and HLA-B51 and -Cw1 class I molecules present some peptides with similar anchor motifs (SYFPEITHI database (40)), the possible HLA-B51 cross-reactivity of D8L$_{112–119}$ ligand was examined. HLA-peptide complex stability assays were performed in which HLA-B51 molecules were stained in the presence of the VACV ligand. In contrast to a control HLA-B51 ligand, the HBV HBc peptide, induction of complexes with the vaccinia D8L$_{112–119}$ peptide was not detected (Fig. 3, *lower panel*). In summary, this viral ligand binds to HLA-E and -Cw1 but not to HLA-A2 or -B51 alleles.

The Viral D8L$_{112–119}$ Peptide Inhibits Cytolysis Mediated by Natural Killer Cells—Because HLA-E/peptide-complexes are recognized by different NK cell receptors mediating either activating or inhibitory signals, we studied whether the binding of D8L$_{112–119}$ ligand to HLA-E alters cytolytic activity of natural killer cells. Thus, 721.221 HLA-E$^+$ cells were incubated with the viral peptide, and a standard ^{51}Cr release assay was performed. As indicated in Fig. 4, incubation of target cells with the D8L$_{112–119}$ peptide reduced their susceptibility to NK3.3-mediated cytotoxicity (65 ± 9% of specific inhibition) to a similar extent as was obtained with the positive control peptide (71 ± 6% of specific inhibition), whereas incubation of 721.221 cells with an irrelevant peptide had no effect on cytotoxic function of NK3.3 cells.

VACV D8L$_{112–119}$ Uses Alternative Interactions to the Anchor Motifs Previously Described for Its Presenting HLA-E Class I Molecule—The crystal structure of HLA-E, in complex with the peptide VMAPRALLL, has been previously described (41). In contrast to classical HLA class I molecules, where the peptides that bind different allotypes are anchored by two primary spec-

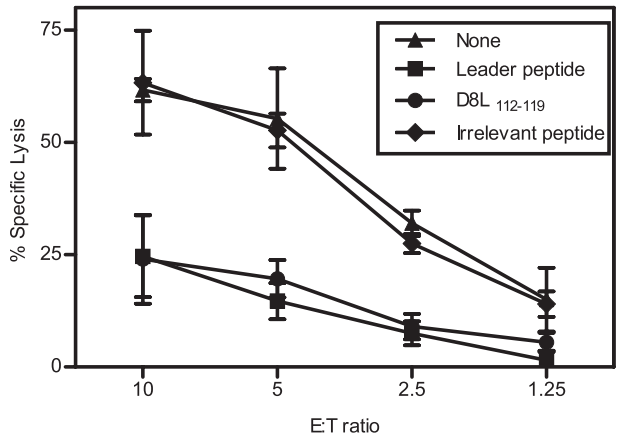


FIGURE 4. **VACV D8L$_{112–119}$ synthetic peptide inhibits HLA-E-mediated cytotoxicity by natural killer cells.** 721.221 target cells prepulsed with 100 μM of the indicated synthetic peptides were tested in a standard cytolytic assay for natural killer cytotoxicity with NK3.3 cells. The leader peptide of HLA (*squares*) and an irrelevant peptide (*diamonds*) were used as positive and negative controls, respectively. The data are the means of three independent experiments ± S.D. ***, significant *p* values ($p < 0.001$) were found between no peptide or irrelevant peptide *versus* leader peptide of HLA or D8L$_{112–119}$.

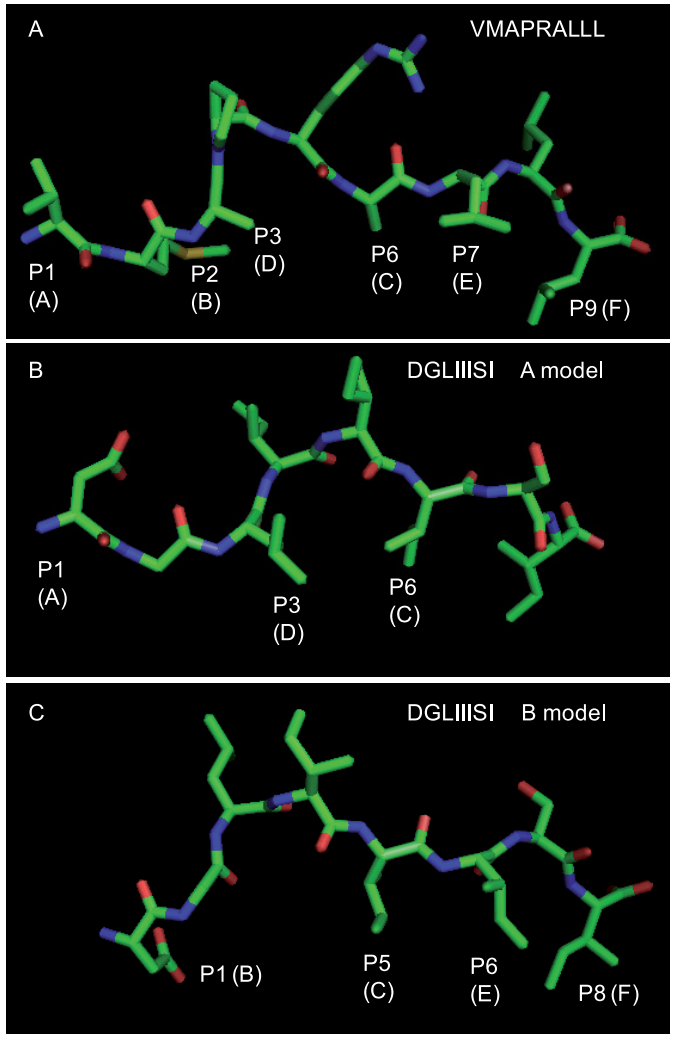


FIGURE 5. **Modeling of HLA-E-bound conformations of VACV D8L$_{112–119}$ peptide.** The backbone atoms of the indicated HLA-E-bound peptides are displayed as *ribbon tubes* (*A*, VMAPRALLL; *B* and *C*, DGLIIISI in two possible conformations: A model and B model, respectively). The atoms are represented by sticks using the following color scheme: *blue*, nitrogen; *red*, oxygen; and *green*, carbon. The peptide residues that interact with the indicated HLA-E pockets are designated. The HLA-E protein is not displayed. The figure was prepared using the PyMOL program.

ificity pockets in the binding groove, HLA-E possesses five main anchor sites at the P2, P3, P6, P7, and P9 positions (Fig. 5*A*). The pockets accommodating the side chains at the P2, P7, and P9 positions are deep, but those at the P3 and P6 positions are relatively shallow. The A pocket interacts with the P1 Val residue (Fig. 5*A*). The P2 and P3 side chains are directed into pockets on opposite sides of the groove to the deep B and the shallow D pockets. The superficial C pocket interacts with the P6 Ala residue, and the P7 residue is accommodated into E pocket. Last, the side chain of P9 is buried in the F pocket.

Modeling of the VACV D8L$_{112–119}$ octamer peptide in complex with HLA-E was based on the existing x-ray structure of the VMAPRALLL-HLA-E complex (Fig. 5). Two alternative conformations with similar HLA-E interaction energies were predicted. The first (A model) keeps the interaction of P1 N-terminal residue with the A pocket but loses contact with the CΩ residue with the F pocket (Fig. 5*B*). In contrast, in the second conformation (B model), the lateral chain of the N-terminal P1 residue is buried in the B pocket, and contacts between the side chain of P8 and the F pocket are conserved (Fig. 5*C*). The P6 residue is an anchor residue in both alternative conformations, although it interacts with different pockets: the C pocket in the A model (Fig. 5*B*) and the E pocket in the B model (Fig. 5*C*). These different models predicted mutually exclusive interactions with either the P3 residue and the D pocket (A model; Fig. 5*B*) or the P5 residue and the C pocket (B model; Fig. 5*C*).

To test the two alternative models, new HLA-peptide complex stability assays were performed using monosubstituted Ala analogues of D8L$_{112–119}$ peptide. Substituting Ala with the P6 Ile residue, which could serve as anchor motif in either of the two conformations, abolished interactions between the HLA and the viral octamer (Fig. 6). Additionally, substituting the P3 Leu with Ala abrogated peptide binding to HLA-E molecule, suggesting that this residue serves as an anchor motif as it does in the A model (Fig. 5*B*). In contrast, exchanging Ala with either P5 Ile or P8 Ile residues, which could be additional anchor residues for HLA molecule in the B model but were absent in the A model as suggested by the modeling of Fig. 5 (*B* and *C*), had no effect on the stabilization of HLA-E molecules (Fig. 6). In summary, the analysis of HLA-D8L$_{112–119}$ interactions using monosubstituted Ala analogues indicated that this viral peptide bound to HLA-E using only the two anchor residues P3 Leu and P6 Ile, consistent with the A model but not consistent with the B model.

DISCUSSION

The results reported here show that the 112–119 octamer derived from the vaccinia D8L protein is efficiently presented by the MHC class I molecule HLA-E using alternative interactions with the anchor motifs previously described for this nonclassical MHC class I molecule. In addition to the its role in the presentation of monomorphic signal peptides derived from the classical HLA class I proteins to the innate receptors (13), HLA-E was identified as a restriction element for bacteria (42,

43) and some viral peptides from EBV (15), HCV (16), HIV (44), and influenza (15). Moreover, most of these viral, but not bacterial, peptides are capable of binding to both classical HLA-A2 and nonclassical HLA-E class I molecules (15, 16), suggesting that HLA-E may bind, at least in part, a repertoire of viral pathogen antigens comparable with HLA-A2 despite the large sequence differences found. Comparison between the HLA-E and HLA-A2 sequences shows 42 amino acid differences in residues located in the peptide binding groove, with 15 conservative and 27 nonconservative changes (Table 1). In our study, the vaccinia ligand showed HLA cross-reactivity with HLA-Cw1 but not HLA-A2 class I molecules. Sequence analysis between HLA-E and HLA-Cw1 class I molecules reveals a lower degree of differences than HLA-A2 with 14 conservative and 20 nonconservative amino acid changes in the residues facing the antigen-binding site (Table 1). Fourteen residues are identical in HLA-E and HLA-Cw1 and are not conserved in the HLA-A2 sequence, but only six of them are facing the peptide binding groove: three in the β sheets (Ser-24, Leu-95, and Trp-97) and three in the α helix (Arg-62, Ala-71, and Glu-152) (Fig. 7). When similar sequence analysis between HLA-E and HLA-B51, which did not cross-react with the D8L viral ligand, was carried out, only four residues located in the peptide binding groove (Ser-24, Ala-71, Leu-95, and Trp-97) were observed to be identical, but they were not conserved in the HLA-A2 or -B51 class I molecules (Table 1 and Fig. 7). All four of these residues match the minimum desetope conserved between HLA-E and HLA-Cw1 but absent in HLA-A2 or -B51 and thus are possibly responsible for the cross-reactivity of the $D8L_{112–119}$ ligand. In addition, although HLA-A2, -B51, and -Cw1 present some peptides with similar P2 and PΩ anchor motifs, overlapping peptide repertoires are not described (SYFPEITHI database (40)). Thus, the cross-reactivity identified in the current report

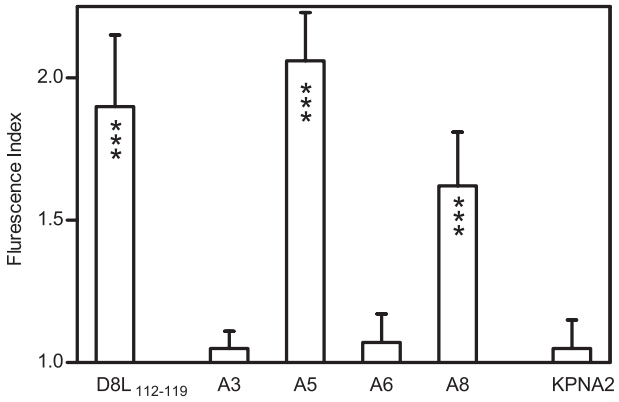


FIGURE 6. **HLA stabilization assay with monosubstituted Ala analogues of VACV $D8L_{112–119}$ synthetic peptide.** Stability at the cell surface of HLA-E on the cell surface of T2 TAP-deficient cells was measured by flow cytometry. The indicated peptides were used at 200 μM. The KPNA2 peptide was used as negative control. The mAb 3D12 was used for staining. The data, calculated as in Fig. 1, are the means of three or four independent experiments. ***, significant p values ($p < 0.001$).

TABLE 1
Polymorphic residues among HLA class I molecules

	Residues[a]																							
	1				10				20				30				40				50				60
HLA-E	G	S	H	S	L	**K**	Y	F	H	T	S	V	S	R	P	G	R	G	E	P	R	F	I	**S**
HLA-Cw1	C			M			F														S			
HLA-A2				M	R		F								A						S			
HLA-B51				M	R		Y	A	M						A						S			
	61				70				80				90				100				110				120
HLA-E	D	R	E	T	R	S	A	R	D	T	A	Q	I	F	R	V	N	L	R	T	L	R	G	Y	Y
HLA-Cw1					Q	K	Y	K	R	Q		T	D		S		N								
HLA-A2		G			K	V	K	A	H	S		T	H		D	G									
HLA-B51			N	Q		K	T	N	T		T	Y		E			I	A	L	R					
	121				130				140				150				160				170				
HLA-E	K	D	Y	L	T	L	N	E	D	L	R	S	W	T	A	V	D	T	A	A	Q	I	S	E	Q
HLA-Cw1				I	A						A														
HLA-A2				I	A	K					A	M													
HLA-B51				I	A			S			A														

	K	S	N	D	A	S	E	A	E	H	Q	R	A	Y	L	E	D	T	C	V	E	W	L	H	K	Y	L	E	K	G	K	E	T	L
HLA-Cw1																																		
HLA-A2																																		
HLA-B51																																		

[a] α1 and α2 HLA-E residues were compared with HLA-Cw1, -A2, or -B51 class I Molecules. Blanks indicate identity with the HLA-E-reference sequence. The identical residues identified between HLA-E and HLA-Cw1 but not HLA-A2 or -B51 molecules (minimum desetope) and proposed as contributing to a shared structural motif that could confer peptide presenting similarities to HLA-E and HLA-Cw1 are in bold type. The sequences are from the IMGT/HLA Database.

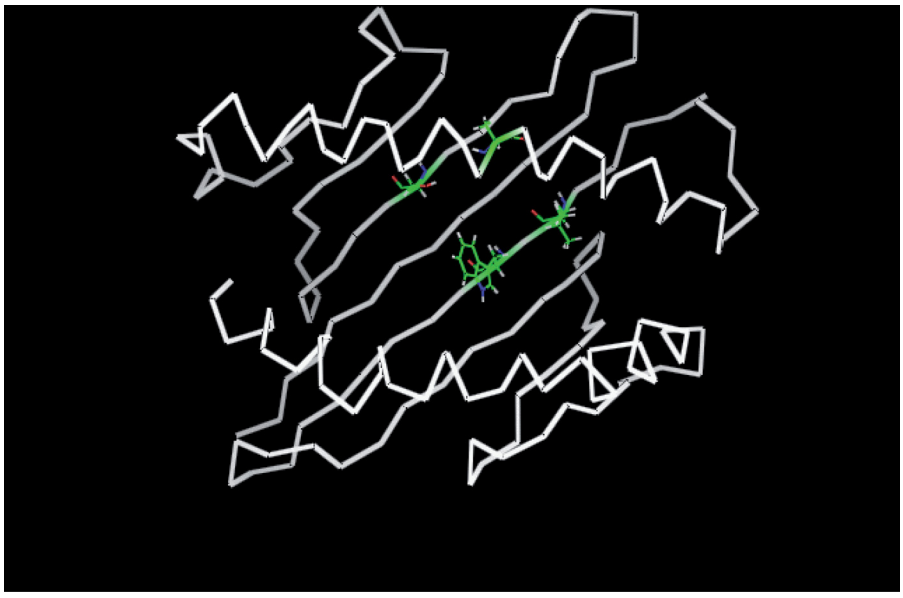


FIGURE 7. **Structural similarities between HLA-E and HLA-Cw1 but not with HLA-A2 or-B51.** The amino acid sequence of the α1 and α2 domains of HLA-E (*white backbone*) was compared with the sequence of the equivalent domains of HLA-A2, -B51, and -Cw1 class I molecules using the same alignment used by Bjorkmann *et al.* (53, 54). The identical residues identified between HLA-E and HLA-Cw1 but not HLA-A2 or -B51 molecules (minimum desetope) and proposed as contributing to a shared structural motif that could confer peptide presenting similarities between HLA-E and HLA-Cw1 are depicted. The atoms of these four residues are represented by sticks using the following color scheme: *blue*, nitrogen; *red*, oxygen; and *green*, carbon. The viral peptide is not displayed. The figure was prepared using the PyMOL program.

between HLA-E and -Cw1, an allele not clustered into HLA-A2 supertype, expands the range of possible HLA-E cross-reactivity and indicates that several other HLA class I viral ligands different from HLA-A2-restricted epitopes could be presented in association with HLA-E. Thus, future studies analyzing the HLA-E peptide repertoire under infection conditions with different viruses are needed.

Some studies have shown cross-reactivity of epitopes between very different MHC class I molecules. Cross-reactivity between multiple HLA-B alleles (HLA-B7, -B27, -B40, -B54, -B55, and -B56) that differ by ~20 residues facing the antigen-binding site has been widely reported (45). Additionally, interspecies cross-reactivity of viral ligands, shared by a human and a rhesus macaque, a rhesus macaque and a mouse, a human and a mouse, and two different chimpanzee MHC class I molecules have been described (46–49). These pairs of cross-reactive MHC molecules are very different and have marked differences in the sequence and structure of the peptide-binding groove. Dual reactivity of $CD8^+$ T cell clones reflected presentation of structurally related peptides by two HLA class I and II molecules: HLA-B27 and HLA-DR2 (50). These findings and the results reported in the current study show the complexity and plasticity of interactions in MHC-peptide complexes.

Our study includes one distinct difference from the previous viral classical and nonclassical HLA cross-reactivity reports (15, 16, 44); the $D8L_{112-119}$ ligand was isolated from TAP-deficient vaccinia virus-infected cells, and thus this viral ligand was naturally processed by a TAP-independent pathway previous to its presentation by HLA-E. Only the HCMV gpUL-40-derived ligand is currently known to assemble with HLA-E via a TAP-independent mechanism (51). This peptide exactly matches the leader sequence peptides of various HLA class I alleles and is able to substitute for the natural leader peptides from HLA-E produced by TAP that are blocked by the protein US6 in HCMV-infected cells (52). Therefore, this TAP-independent antigen presentation was previously reported as a viral mechanism to bypass the normal HLA-E loading system that evolved to occlude NK cell recognition of infected cells, whereas most HCMV epitopes remain in the cytosol without any possibility of entering the endoplasmic reticulum. Unlike this previously described tolerogenic peptide, $D8L_{112-119}$ could be recognized by $CD8^+$ T cells in the same manner as vaccinia virus-encoded HLA-A2-restricted epitopes generated in the same TAP-deficient infected cells (17), allowing it to contribute to host defense against viral infection. Lastly, the lack of polymorphism of the HLA-E gene in humans suggests that $D8L_{112-119}$ could be a universal epitope, requiring future studies to understand the HLA-E-restricted response of this viral peptide.

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Role of Metalloproteases in Vaccinia Virus Epitope Processing for Transporter Associated with Antigen Processing (TAP)-independent Human Leukocyte Antigen (HLA)-B7 Class I Antigen Presentation*[S]

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Background: Individuals with nonfunctional transporter associated with antigen processing (TAP) present HLA class I ligands generated by TAP-independent processing pathways.
Results: Different subsets of metalloproteinases generate two vaccinia-derived TAP-independent epitopes.
Conclusion: Various proteolytic systems contribute to the antiviral cellular immune response, thereby facilitating immunosurveillance.
Significance: This may explain why TAP-deficient individuals live normal life spans without any increased susceptibility to viral infections.

The transporter associated with antigen processing (TAP) translocates the viral proteolytic peptides generated by the proteasome and other proteases in the cytosol to the endoplasmic reticulum lumen. There, they complex with nascent human leukocyte antigen (HLA) class I molecules, which are subsequently recognized by the CD8$^+$ lymphocyte cellular response. However, individuals with nonfunctional TAP complexes or tumor or infected cells with blocked TAP molecules are able to present HLA class I ligands generated by TAP-independent processing pathways. Herein, using a TAP-independent polyclonal vaccinia virus-polyspecific CD8$^+$ T cell line, two conserved vaccinia-derived TAP-independent HLA-B*0702 epitopes were identified. The presentation of these epitopes in normal cells occurs via complex antigen-processing pathways involving the proteasome and/or different subsets of metalloproteinases (amino-, carboxy-, and endoproteases), which were blocked in infected cells with specific chemical inhibitors. These data support the hypothesis that the abundant cellular proteolytic systems contribute to the supply of peptides recognized by the antiviral cellular immune response, thereby facilitating immunosurveillance. These data may explain why TAP-deficient individuals live normal life spans without any increased susceptibility to viral infections.

Newly synthesized viral proteins are recognized constantly by CD8$^+$ lymphocytes as short peptides bound to human leukocyte antigen (HLA) class I molecules at the cell surface of infected cells (1). Proteolysis by the proteasome and other cytosolic proteases generates most of the peptides presented by HLA class I molecules. These peptides are transported into the endoplasmic reticulum (ER)[2] by the transporter associated with antigen processing (TAP), and subsequent N terminal trimming by the metallo-aminoproteases ERAP1 and −2 is often required (2, 3). Viral peptides assembled with newly synthesized β_2-microglobulin and HLA class I heavy chain generate stable peptide-HLA complexes that are exported to the cell membrane (reviewed in Ref. 4).

Mutations in the TAP genes that generate nonfunctional TAP complexes have been described in both humans (5) and mice (6). This HLA class I deficiency implies a reduced functional CD8$^+$ population but does not correlate with any increased susceptibility to viral infections or neoplasms. Thus, TAP-deficient patients live normal life spans with only a limited susceptibility to chronic respiratory bacterial infections. Therefore, their immune systems must be reasonably efficient, and antibodies, NK cells, CD8$^+$ $\gamma\delta$ T cells, and the reduced cytolytic CD8$^+$ $\alpha\beta$ T subpopulation that is specific for TAP-independent antigens may all contribute to immune defenses that protect against severe viral infections in these individuals. Some viruses block TAP expression or function to prevent cellular immune responses from identifying infected cells (reviewed in Ref. 7). Therefore, TAP-independent pathways must be important for killing cells infected with these viruses. TAP-independent pathways of antigen presentation of various pathogenic epitopes by MHC class I molecules have previously been reported (reviewed in Refs. 7–9).

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[2] The abbreviations used are: ER, endoplasmic reticulum; BFA, brefeldin A; BEN, benzyl succinyl acid; BES, bestatin; CTL, cytotoxic T lymphocyte; ICS, intracellular cytokine staining; LC, lactacystin; LeuSH, leucine thiol; PHE, 1,10-phenanthroline; TAP, transporter associated with antigen processing; VACV, vaccinia virus; ERAP, ER aminopeptidase.

Cross-protective vaccination with orthopoxviruses, first with an empirically developed vaccine against cowpox virus and later through the massive worldwide administration of vaccinia virus (VACV), achieved the eradication of smallpox, a pandemic disease caused by variola major virus (10). The role of cellular responses in this cross-protection is well documented (11, 12). The cowpox protein CPXV12 inhibits peptide translocation by TAP, thereby interfering with MHC class I-peptide complex formation (13). Thus, the identification of TAP-independent epitopes conserved among orthopoxviruses could be relevant to the study of the mechanisms of early empirical vaccination against smallpox disease performed with cowpox virus. In this study, using a TAP-independent polyclonal vaccinia virus-polyspecific $CD8^+$ T cell line, we identified two VACV-derived TAP-independent epitopes that are conserved among the Orthopoxviridae family, including cowpox virus.

EXPERIMENTAL PROCEDURES

Mice—H-2 class I double knock-out HLA-B*0702 transgenic mice (14) were bred in our animal facilities in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Spanish "Comisión Nacional de Bioseguridad" of the "Ministerio de Medio Ambiente y Medio Rural y Marino" (accreditation number 28079–34A). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Institute of Health "Carlos III" (Permit Number: PI-283). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Cell Lines—The mouse cell lines RMA (TAP-positive) and RMA-S (TAP-negative) stably expressing HLA-B*0702 $\alpha1\alpha2$ domains plus the mouse H-$2D^b$ $\alpha3$ transmembrane and cytoplasmic domains have been previously described (14). All cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 5 μM β-mercaptoethanol.

Synthetic Peptides—Peptides were synthesized in a peptide synthesizer (model 433A; Applied Biosystems, Foster City, CA) and purified by reverse-phase HPLC. The correct molecular mass of the peptides was established by MALDI-TOF MS, and their correct composition was verified by quadrupole ion trap micro-HPLC.

Inhibitors—Brefeldin A (BFA) and all protease inhibitors were purchased from Sigma, with the exception of leupeptin (Amersham Biosciences), pepstatin (Roche Applied Science), benzyloxycarbonyl-VAD-fluoromethyl ketone (Enzyme System Products, CA), and lactacystin (Dr. E. J. Corey, Harvard University). The specificity and activity of inhibitors used in this study are summarized in Table 1. As a control for the activity of protease inhibitors that do not block antigen presentation, RMA-HLA-A*0201 cells (1×10^8) were disrupted by sonication for 15 min at 4 °C and centrifuged as reported previously (15). A supernatant aliquot corresponding to 1×10^7 cells was directly frozen (nondegraded control). Equivalent aliquots were incubated in the presence of individual inhibitors at 200 μM, and digestion by cellular proteases was allowed for 5 days at 37 °C in PBS. Inhibitors were renewed daily. A sample incubated without inhibitors was taken as the degraded control. After SDS-PAGE separation and Coomassie Blue staining of these samples, the overall protein content of each lane was quantitated by densitometry with the TINA 2.09e program (Isopenmessgeräte, GmbH, Germany). Percent inhibition of protein degradation caused by each inhibitor was calculated as follows: 100 × (sample with inhibitor − degraded)/(nondegraded − degraded).

Ligand Prediction—The on-line program SYFPEITHI was used to predict HLA-B*0702-specific ligands of VACV as described previously (16).

Ex Vivo Intracellular Cytokine Staining (ICS)—Intracellular cytokine staining assays were performed as described previously (17). Spleen cells were obtained from HLA-B*0702 transgenic mice at 7 days (acute response) or up to 30 days (memory response) post-intraperitoneal (intraperitoneal) infection with 1×10^7 pfu of VACV-WR as described previously (18). After harvest, cells were stimulated for 2 h with RMA HLA-B*0702 cells infected with VACV-WR and incubated for 3 h in the presence of 5 μg/ml BFA. Later, cells were incubated with FITC-conjugated anti-CD8 mAb (ProImmune, Oxford, UK) for 30 min at 4 °C, fixed with Intrastain kit reagent A (Dako-Cytomation, Glostrup, Denmark), and incubated with phycoerythrin-conjugated anti-IFN-γ mAb (Pharmingen) in the presence of Intrastain kit permeabilizing reagent B for 30 min at 4 °C. Results were acquired on a FACSCanto flow cytometer (BD Biosciences) and analyzed using CellQuest Pro 2.0 software (BD Biosciences).

T Cell Lines, Cytotoxicity Assays, and ICS—The TAP-independent polyclonal VACV-polyspecific $CD8^+$ T cell line was generated by immunizing mice intraperitoneally with 1×10^7 pfu of VACV-WR. Splenocytes from immunized mice were restimulated weekly *in vitro* with mitomycin C-treated VACV-infected RMA or RMA-S HLA-B*0702 cells as antigen-presenting cells. Also, uninfected mitomycin C-treated spleen cells of allogeneic BALB/c (H-2^d haplotype), C3H (H-2^k haplotype), and SJL (H-2^s haplotype) mice were alternately used as feeder cells. This allogeneic system prevents cross-presentation of TAP-dependent HLA-B*0702-restricted peptides in the cell culture. The $CD8^+$ T cell line was re-stimulated with VACV-infected RMA-S HLA-B*0702 cells and was cultured in α-minimal essential medium supplemented with 10% FBS and 1% β-mercaptoethanol and was used after five re-stimulations as effector cells in standard 4-h cytolytic assays (18) or ICS staining similarly to *ex vivo* ICS.

Polyclonal SIINFEKL or VACV peptide-monospecific CTLs were generated by immunizing mice intraperitoneally with 1×10^7 pfu of VACV-OVA(257–264) encoding the miniprotein MSIINFEKL or VACV-WR as described previously (15, 19), respectively. Splenocytes from immunized mice were restimulated *in vitro* with mitomycin C-treated spleen cells pulsed with 10^{-6} M of the respective peptide and cultured in α-minimal essential medium supplemented with 10% FBS, 1×10^{-7} M peptide, and 1% β-mercaptoethanol. Recombinant human interleukin-2 was generously provided by Hoffmann-La Roche for the long term propagation of all $CD8^+$ T cell lines. ICS assays to detect the recognition of infected cells by polyclonal $CD8^+$ T cell lines were performed as described previously (17). $CD8^+$ T cell lines were stimulated for 4 h with RMA HLA-B*0702 target cells that had been infected with VACV or

VACV-OVA(257–264) overnight and in the presence of 5 μg/ml BFA. When protease inhibitors were used, all drugs were added 15 min before the virus and kept at a 5-fold higher concentration during the 1-h adsorption period than that used throughout the infection. After the virus inoculums were washed, the inhibitors were kept at the concentrations indicated for the individual experiments. The inhibitors were not toxic at the indicated concentrations because they did not affect antigen presentation of either the J6R(303–311) or D1RL((807–817)) epitopes (see below) or the VACV infection when the Omnitope antiserum with specificity for VACV proteins from purified virions was used (ViroStat Inc., Portland, ME) (supplemental Table 1). ICS with polyclonal $CD8^+$ T cell lines was performed similarly to *ex vivo* ICS. The percentage of specific inhibition obtained by the addition of the inhibitors was calculated as shown in Equation 1,

$$\%\ \text{specific inhibition} = 100 - \frac{((\text{ICS VACV} + \text{inhibitor}) - (\text{ICS without infection}))}{(\text{ICS VACV} - \text{ICS without infection})} \times 100 \quad \text{(Eq. 1)}$$

Statistical Analysis—To analyze statistical significance, an unpaired Student *t* test was used. p values < 0.01 were considered to be significant.

RESULTS

*Identification of Two VACV-derived TAP-independent HLA-B*0702 Epitopes*—Spleen cells from HLA-B*0702 transgenic mice were primed with VACV and re-stimulated with the TAP-negative RMA-S cell line transfected with HLA-B*0702 to specifically recognize VACV-infected target cells. We observed 45% specific lysis *versus* 1% without virus in a standard ^{51}Cr-release assay. By intracellular cytokine staining (ICS) assays, we found that 32% of IFN-γ secreting cells had a specific response *versus* 1% without virus. Later, this $CD8^+$ cell line was used to identify TAP-independent epitopes with target cells previously pulsed with the VACV synthetic peptides previously reported as HLA-B*0702 epitopes identified from either HLA-B*0702 transgenic mice (A34R(82–90), D1R(808–817), and J2R(116–124)) (20) or human vaccines (AC1L(97–106), D1R(686–694), F4L(6–14), and J6R(303–311)) (21, 22). In addition, 14 HLA-B7 potential ligands from a VACV proteome-based *in silico* prediction of high binding were also included in the study to identify new epitopes (supplemental Table 2). In a preliminary ^{51}Cr-release assay, only two synthetic peptides, D1R(808–817) and J6R(303–311), were recognized by the TAP-independent CTL cell line (Fig. 1*A*, *arrows*). Additional ICS experiments with all synthetic peptides confirmed that only the D1R(808–817) and J6R(303–311) peptides specifically stimulated the production of IFN-γ in the $CD8^+$ T cells specific for VACV ligands (Fig. 1*B*). Fig. 2 shows that D1R(808–817) peptide was recognized 10-fold less efficiently in TAP-deficient cells *versus* TAP-sufficient cells. Both viral ligands were conserved among the *Orthopoxviridae* family, including cowpox virus (NCBI data base (/blast.ncbi.nlm.nih.gov). In summary, these results indicate that two conserved TAP-independent HLA-B7 epitopes were present in the TAP-deficient VACV-infected cells.

*Partial Interspecies Overlap in the $CD8^+$ Repertoire against HLA-B*0702 Viral Epitopes*—Previously, the D1R(808–817) viral epitope was immunogenic in the HLA transgenic mouse model (20), whereas the J6R(303–311) peptide was recognized by peripheral blood mononuclear cells of an HLA-$B7^+$ donor immunized with VACV (21). No interspecies overlap in the $CD8^+$ repertoire against these two VACV epitopes or the other five HLA-B*0702 epitopes has been reported (20–22). Because the HLA-B*0702 transgenic mice used in this study (14) have a different origin from those used the previous study (20), the VACV-specific $CD8^+$ acute and memory responses in our HLA-B7 transgenic model was evaluated using *ex vivo* ICS assays. A strong acute response ($6.1 \pm 0.4\%$ of IFN-γ-secreting cells) specific for the D1R(808–817) synthetic peptide was detected (Fig. 3). The A34R(82–90) and D1R(686–694) viral peptides were also immunogenic, with 3.0 ± 0.7 and $1.2 \pm 0.4\%$ $CD8^+$ IFN-γ^+ cells, respectively. Additionally, a small fraction of VACV-specific $CD8^+$ T lymphocytes recognized the J6R(303–311) peptide ($0.3 \pm 0.05\%$ of IFN-γ^+ cells, Fig. 3). When the VACV memory response was analyzed (Fig. 3, *filled bars*), an epitope hierarchy similar to the acute response was found, except that the percentage of IFN-γ secreting cells was slightly higher with the J6R(303–311) peptide ($0.7 \pm 0.2\%$) than with the D1R(686–694) epitope ($0.5 \pm 0.1\%$). None of the other 17 VACV peptides tested (for list see Fig. 1 and supplemental Table 1) stimulated the production of IFN-γ in the VACV-specific $CD8^+$ T cell acute or memory response (data not shown). Thus, two epitopes from both previously described transgenic mouse models (D1R(808–817) and A34R(82–90)) and human donors (D1R(686–694) and J6R(303–311)) are responsible for this specific $CD8^+$ response against VACV. The same *ex vivo* epitope hierarchy was also found using a TAP-dependent polyclonal VACV-polyspecific $CD8^+$ T cell line generated by re-stimulation *in vitro* with mitomycin C-treated VACV-infected RMA (TAP^+) HLA-B*0702 cells as antigen-presenting cells (data not shown). By contrast, in the previous study with the HLA transgenic model, the epitope hierarchy was A34R(82–90) > J2R(116–142) > D1R(808–817) (20). These results show both the quantitative and the qualitative differences between the two HLA-B*0702 transgenic mouse models available, although only the $CD8^+$ response of the HLA-B7 model used in our study showed partial overlap in $CD8^+$ repertoire with the study of human vaccines (21). In addition, these data show that half of the HLA-B7-restricted viral epitopes detected in a normal TAP-dependent T cell response could also be presented in a TAP-independent manner in the HLA-B7 transgenic model.

*Endogenous Processing of TAP-independent HLA-B*0702 Epitopes*—To study all antigen-processing pathways involved in the endogenous generation of the D1R(808–817) and J6R(303–311) viral epitopes, polyclonal $CD8^+$ T cell lines monospecific for the two TAP-independent HLA-B7 viral epitopes were generated. Later, we investigated the presentation of these epitopes to respective specific CTLs in the presence of diverse protease inhibitors in VACV-infected TAP-proficient cells. To test whether these HLA-B7-restricted epitopes

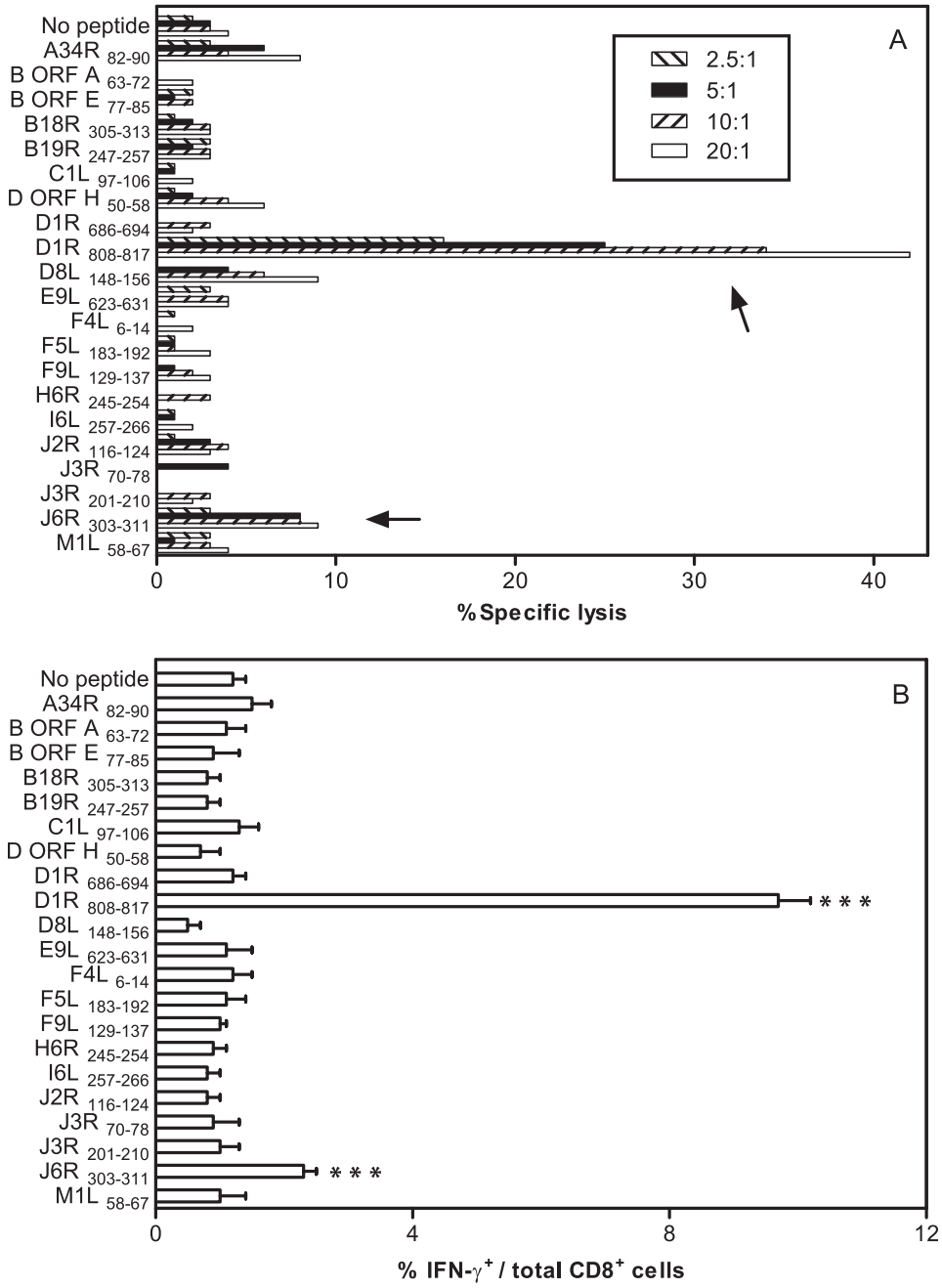

FIGURE 1. **Recognition of HLA-B7-restricted synthetic peptides by a VACV-specific TAP-independent CD8$^+$ T cell line.** RMA-HLA-B*0702 target cells pre-pulsed with 10^{-5} M of the indicated VACV-synthetic peptides were tested in a standard cytolytic assay (*A*) or by ICS for CD8$^+$ T cell activation (*B*) with VACV-specific CD8$^+$ T cells. The VACV-specific CD8$^+$ T cells were obtained from HLA-B*0702-transgenic mice immunized with VACV up to 30 days prior and restimulated *in vitro* with the TAP-negative RMA-S cell line transfected with HLA-B*0702. *Arrows* in *A* indicate the synthetic peptides detected with 2-fold higher specific lysis than the negative control (no peptide) in three *E*/*T* ratios. The data are the mean of four independent experiments ± S.D. (*B*). Significant *p* value, ***, $p < 0.001$.

require endogenous processing, we analyzed their presentation in the presence of BFA. This drug blocks class I export beyond the cis-Golgi compartment (23, 24), preventing the surface expression of newly assembled class I-peptide complexes of endogenous origin (Table 1 summarizes the specificity of all inhibitors used in this study). We observed complete inhibition of specific secretion of IFN-γ in the two specific CD8$^+$ T cell lines by the addition of BFA during infection (Fig. 4), demonstrating that these epitopes were indeed generated from proteins endogenously processed in VACV-infected cells.

*Proteasome Inhibitor Differentially Affects the Antigen Presentation of TAP-independent HLA-B*0702 Viral Ligands*—Lactacystin (LC), a bacterial metabolite (Table 1) (25–27), was used to study the role of the proteasome in the presentation of these epitopes. LC partially blocks (45 ± 15%) the specific recognition of target cells infected with VACV by J6R(303–311)-specific CD8$^+$ T cells (Fig. 4). By contrast, in the same experiment, this drug had no effect on the presentation of the D1R(808–817) epitope (3 ± 12%) (Fig. 4). We observed complete inhibition of infected cell recognition by another VACV-specific

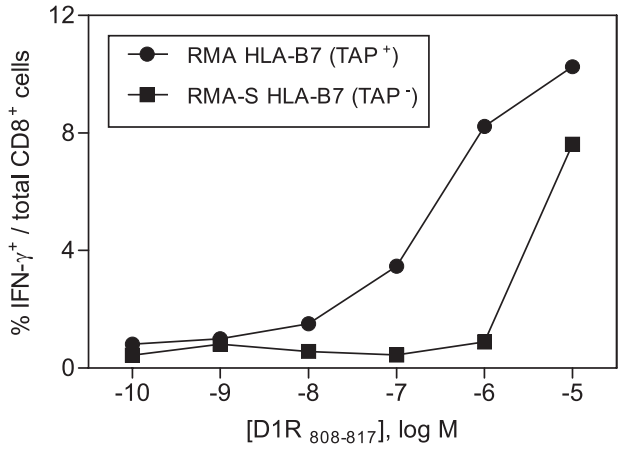

FIGURE 2. **Recognition of TAP$^+$ and TAP$^-$ cell lines by VACV-specific CD8$^+$ T lymphocytes.** Recognition by ICS for CD8$^+$ T cell activation of titration curves of D1R(808–817) synthetic peptide in HLA-B*0702 TAP$^+$ (*RMA*, *circles*) and TAP$^-$ (*RMA-S*, *squares*) cells. The VACV-specific CD8$^+$ T cells were obtained as Fig. 1. Results are the mean of three experiments.

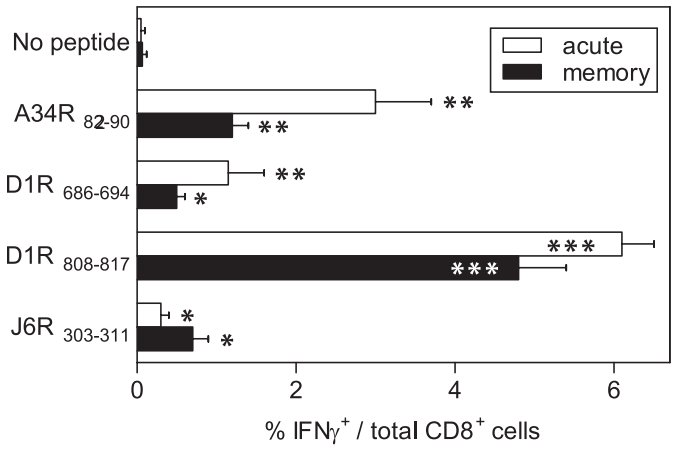

FIGURE 3. **Immunogenicity of VACV-derived HLA-B*0702-restricted peptides in HLA-B*0702 transgenic mice.** RMA-HLA-B*0702 target cells prepulsed with the indicated VACV synthetic peptides in Fig. 1 were analyzed by ICS for CD8$^+$ T cell activation with VACV-specific splenocytes obtained from HLA-B*0702 transgenic mice immunized 7 days (*acute* response, *open bars*) or up to 30 days *(memory*, *closed bars*) post-infection. The results are calculated as the mean of three or four independent experiments ± S.D. Significant *p* values are as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

TAP$^+$ CD8$^+$ T cell line with LC treatment (96 ± 4%),[3] indicating that LC-mediated inhibition of proteasome activity is not absolutely required for antigen processing of the D1R(808–817) epitope. This further suggests that the proteasome partially contributes to the generation of the J6R(303–311) peptide in infected cells.

*A Metallopeptidase Inhibitor Specifically Blocks the Recognition of HLA-B*0702 Epitopes*—To characterize proteases distinct from proteasomes that may contribute to the processing of HLA-B*0702-restricted ligands, experiments with several specific protease inhibitors were performed. Leupeptin (28), pepstatin (28, 29), 1,10-phenanthroline (PHE) (29, 30), and E64 (31) inhibitors were initially tested because they are specific for different protease families (Table 1) and cover a wide range of protease classes. Puromycin (32) has also previously been suggested to generate pathogen-derived peptides; thus, the possible role of this enzyme in endogenous presentation of TAP-independent viral epitopes was studied using a specific inhibitor (Table 1). Four of five inhibitors had no effect on the specific recognition of target cells infected with VACV with the two specific CD8$^+$ T cell lines tested (Fig. 5). Thus, the enzymes inhibited by these drugs are not formally involved in the generation of TAP-independent ligands.

In contrast, PHE inhibited the recognition of infected cells by J6R(303–311) (42 ± 12%)- and D1R(808–817) (72 ± 20%)-specific CD8$^+$ T cells (Fig. 5). We wanted to exclude the possibility that the inhibitory effect of PHE was due to toxic effects on target cells or on VACV replication rather than to a specific block of the respective proteases. To this end, experiments similar to those shown in Fig. 5 were performed in parallel using VACV-OVA(257–264)-infected target cells. These infected cells were efficiently recognized by the SIINFEKL-specific CD8$^+$ T cell line. and no inhibition was detected (10 ± 4%, see Fig. 5). These data indicate that inhibition of specific recognition in both CD8$^+$ T cell lines by addition of PHE is formally due to specific blockage of the specific proteases not to a block in VACV replication (see also supplemental Table 1). In summary, these results indicate that either a metallopeptidase or different metalloproteases are involved in the generation of these two HLA-B*0702 epitopes.

*Metallocarboxyprotease and Metalloaminoproteases but Not ERAP Are Differentially Involved in the Generation of TAP-independent HLA-B*0702 Epitopes*—A variety of functional metallopeptidases are located in the cytosol or in other compartments related to the MHC class I presentation pathway, such as the ER and the trans-Golgi network (reviewed in Ref. 33). Any of these enzymes may play a role in the endogenous pathway of antigen processing. These enzymes can be grouped into aminopeptidases, endopeptidases, carboxypeptidases, and carboxydipeptidases, among others, based on their respective cleavage mechanism (reviewed in Ref. 34). Some of these groups can be distinguished by the use of different specific inhibitors (summarized in Table 1).

To more specifically identify the metallopeptidase group involved in antigen processing of both D1R(808–817) and J6R(303–311) viral peptides, VACV-infected target cells were treated with specific subfamily inhibitors (Table 1). The caspase-1-specific inhibitor benzyloxycarbonyl-VAD was also included due to the sensitivity of this cysteine protease to PHE (30). None of the inhibitory compounds used, except for leucine thiol (LeuSH), prevented antigen presentation of VACV-infected cells to the CD8$^+$ T cell line specific for the J6R(303–311) viral peptide (Fig. 6, *open bars*). When similar experiments were carried out with D1R(808–817)-specific CD8$^+$ T cells, complete blockage of antigen recognition (84 ± 11%) was also detected in the presence of the LeuSH inhibitor. The inhibitory effect of LeuSH was specific to these two viral epitopes because the recognition of VACV-OVA(257–264) cells by the SIINFEKL-specific CD8$^+$ T cell line was not abrogated in the presence of this compound (12 ± 10%, see Fig. 6). Thus, these data implicate ERAP or other metallo-aminopeptidases in the generation of the two VACV epitopes studied.

[3] E. Lorente and D. López, manuscript in preparation.

TABLE 1
Specificity and activity of the inhibitors used in this study

Inhibitor	Abbreviation	Specificity	Reference	Concentration	% inhibition of degradation[a]
Brefeldin A	BFA	Vesicle transport	23, 24	5 μg/ml	ND
Lactacystin	LC	Proteasome	25, 26	10 μM	ND
Leupeptin	LEU	Trypsin-like proteases and cysteine proteases	28	100 μM	38 ± 18
Pepstatin	PEP	Aspartic proteases	28, 29	100 μM	50 ± 5
1,10-Phenanthroline	PHE	Metalloproteases and caspase-1	29, 30	50 μM	ND
E64	E64	Cysteine proteases C1	31	100 μM	ND
Puromycin	PUR	Dipeptidyl-peptidase II and PSA	61	0.5 μg/ml	ND
Captopril	CAP	ACE and ACE-like proteases	29	100 μM	25 ± 2
Benzyl succinyl acid	BEN	Metallo-carboxypeptidases A and B	29	100 μM	−10 ± 8
Bestatin	BES	Most of metallo-aminopeptidases	29	50 μM	ND
Phosphoramidon	PHO	All bacterial metallo-endopeptidases but few of mammalian origin	29, 62	100 μM	15 ± 4
Leucine thiol	LeuSH	Metallo-aminopeptidases including ERAP	57	30 μM	ND
Benzyloxycarbonyl-VAD-fluoromethyl ketone	Z-VAD-fmk	Caspases	63	100 μM	ND[b]

[a] Activity of these inhibitors was measured as their ability to prevent proteolytic degradation in cellular extracts as in Ref. 15. The amount of protein still present after incubation in the case of the degraded control sample was considered 0% inhibition of degradation, and the nondegraded sample was taken as 100% inhibition. Data are means of two independent experiments. Negative values indicate that there was enhanced degradation in the presence of the compound. ND indicates not done.
[b] The compound was found to block apoptosis (data not shown).

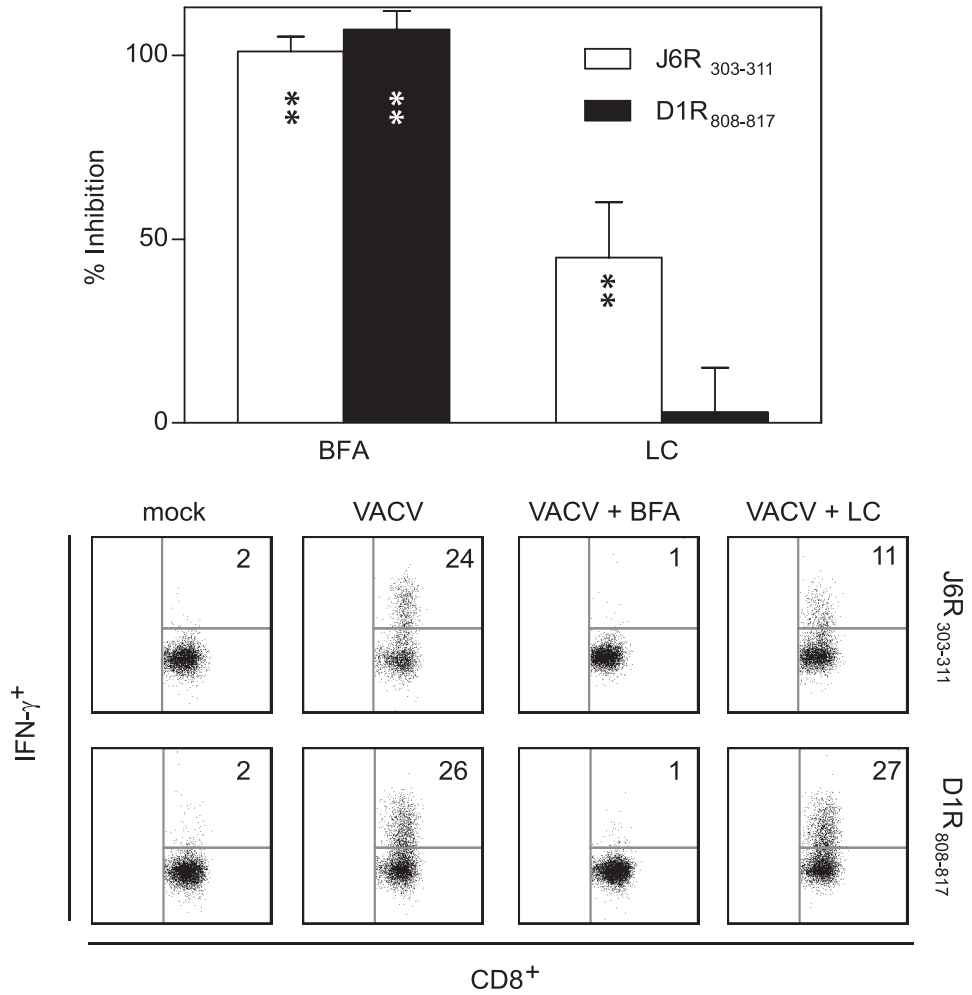

FIGURE 4. **Effect of BFA and a proteasome inhibitor on the recognition of the J6R(303–311) or the D1R(808–817) viral epitopes.** RMA-HLA-B*0702 target cells infected for 16 h with VACV at a multiplicity of infection of 40 plaque-forming units/cell were treated with BFA or LC. An ICS assay was used to test for recognition by the J6R(303–311)- (*open bars*) or D1R(808–817)-specific (*closed bars*) $CD8^+$ T cell lines. The data are expressed as percentage of inhibition ± S.D. as determined by ICS in the presence of the indicated inhibitors and are means of three to four independent experiments. Significant *p* value, **, $p < 0.01$. Representative ICS assays with J6R(303–311)- and D1R(808–817)-specific $CD8^+$ T cell lines were depicted in the *middle* and *bottom panels*, respectively. The percentages of IFN-γ-expressing $CD8^+$ T cells are indicated in each dot plot.

In addition, as shown in Fig. 6, a partial but specific blockage of the endogenous processing of the D1R(808–817) epitope by either the metalloaminoprotease inhibitor bestatin (BES) or the metallocarboxypeptidase inhibitor benzyl succinyl acid (BEN) was detected (44 ± 15% of specific inhibition with BES and 42 ± 16% with BEN). Thus, both metalloaminoprotease and metal-

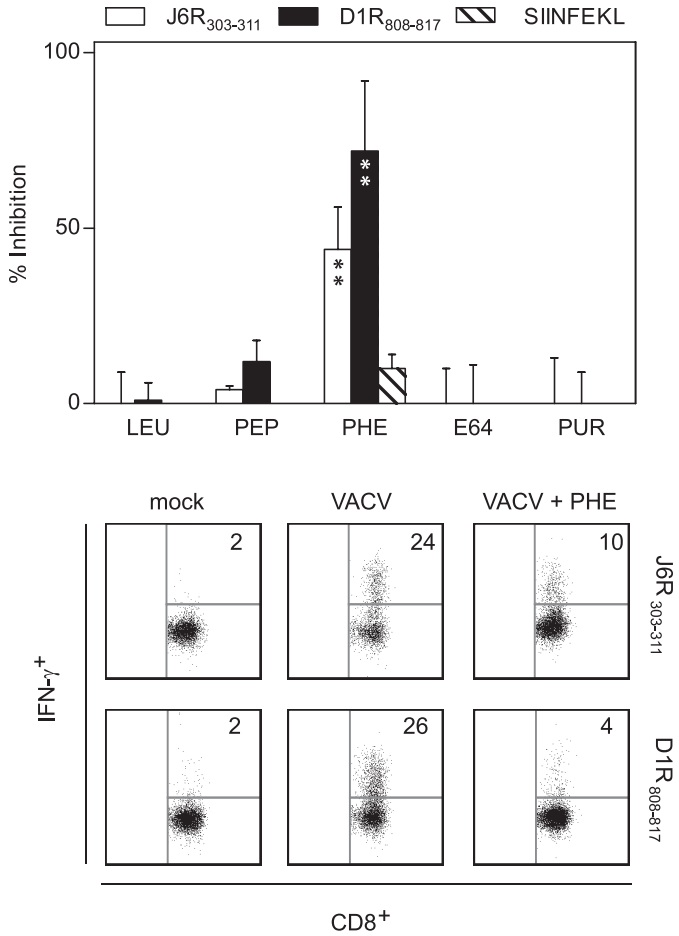

FIGURE 5. **Recognition of VACV-infected cells by J6R(303–311)- or D1R(808–817)-specific CD8$^+$ T cells in the presence of general protease inhibitors.** Cells infected as described in Fig. 4 were treated with leupeptin (*LEU*) (trypsin-like and cysteine protease inhibitor), pepstatin (*PEP*) (aspartic protease inhibitor), PHE (metallopeptidase inhibitor), E64 (cysteine protease C1 inhibitor), or puromycin (*PUR*) (dipeptidyl-peptidase II and PSA), as indicated, before the ICS assay. J6R(303–311)- (*open bars*), D1R(808–817)- (*closed bars*), or SIINFEKL-specific (*hatched bars*) CD8$^+$ T cell lines were used. The percentage of specific inhibition was calculated as in Fig. 4. The data are means ± S.D. of three or four independent experiments. Significant *p* value, **, $p < 0.01$. Representative ICS assays with J6R(303–311)- and D1R(808–817)-specific CD8$^+$ T cell lines were depicted in the medium and bottom panels, respectively. The percentages of IFN-γ-expressing CD8$^+$ T cells are indicated in each dot plot.

locarboxyproteases are involved in the antigen processing of the D1R(808–817) epitope.

J6R(303–311) Epitope May Be Processed in Parallel Either by Proteasomes or by Metallopeptidases Independently—The inhibition of antigen recognition by either LC (Fig. 4) or PHE (Fig. 5) indicates that both proteasomes and metalloproteases are involved in the antigen presentation of the J6R(303–311) epitope. The identical partial inhibition of VACV-infected cell recognition detected with both reagents (45 ± 15 and 42 ± 12% in the presence of LC or PHE, respectively) is compatible with two possible explanations. First, this epitope could be processed by proteasomes and metallopeptidases in a sequential pathway, and other uncharacterized proteases may be responsible for the other half of the antigen. Alternatively, these epitopes could be processed in parallel by proteasomes or by metallopeptidases independently, meaning that these two antigen-processing pathways would need to be inhibited at the same time to fully abrogate the specific recognition by J6R(303–311)-specific CD8$^+$ T cells. To test these hypotheses, the effects of the combination of both inhibitors on antigen presentation in vaccinia-infected cells were analyzed. A total block of presentation (97 ± 3%) was observed in target cells treated simultaneously with LC and PHE (Fig. 7). The inhibitory effect of LC and PHE was specific to these two viral epitopes because the recognition of VACV-OVA(257–264) cells by the SIINFEKL-specific CD8$^+$ T cell line was not abrogated in the presence of these drugs (5 ± 10%, see Fig. 7). These results demonstrate that proteasomes and metallopeptidases are involved in two different antigen-processing pathways that contribute independently to the presentation of the J6R(303–311) epitope.

Sequential Cleavage by Amino- and Carboxy-metallopeptidases Is Involved in Antigen Processing of the D1R(808–817) Epitope—Like the J6R(303–311) epitope, the partial block of D1R(808–817) epitope recognition detected in the presence of BES or BEN (44 ± 15 and 42 ± 16%, respectively) is compatible with either sequential cleavage by amino- and carboxy-metallopeptidases or the activity of these enzymes in two different antigen-processing pathways to produce the D1R(808–817) epitope. The incubation of VACV-infected target cells with a mixture of both reagents produced a partial inhibition of antigen presentation (46 ± 4%, see Fig. 7), similar to two single inhibitors (Fig. 6). The inhibitory effect of the two metallopeptidase inhibitors was specific to the two viral epitopes because the recognition of VACV-OVA(257–264) cells by the SIINFEKL-specific CD8$^+$ T cell line was not abrogated in the presence of BEN plus BES (7 ± 9%, see Fig. 7). Thus, both metalloaminoprotease and metallocarboxyproteases contribute to D1R(808–817) epitope cleavage in the same antigen-processing pathway. In addition, another uncharacterized protease is responsible for the BEN plus BES-resistant antigen processing detected with D1R(808–817)-specific CD8$^+$ T cells.

Diversity of Proteases Involved in Antigen Recognition of Vaccinia HLA-B7-restricted Epitopes—Table 2 summarizes the various inhibition patterns for antigen recognition of the J6R(303–311) and D1R(808–817) epitopes obtained with the drugs used in this study (Table 1). The inhibition observed with BFA indicates that both viral peptides were endogenously processed. The block with LC and PHE indicates that both proteasomes and metalloproteases are used in the processing of the J6R(303–311) epitope. Also, the LeuSH inhibitor impaired antigen recognition of target cells by J6R(303–311)-specific CD8$^+$ T cells. The second epitope, D1R(808–817), shows a different inhibition pattern. LC had no effect on the presentation of this epitope, and thus, LC-mediated inhibition of proteasome activity is not absolutely required for antigen processing of the D1R(808–817) epitope. By contrast, PHE, BEN, BES, and LeuSH significantly decreased antigen presentation of the D1R(808–817) epitope; thus, a variety of metalloproteases are involved in the generation of this TAP-independent epitope.

DISCUSSION

This study was undertaken to identify TAP-independent HLA-B*0702 epitopes from the vaccinia virus and to study their antigen presentation pathways. First, our results indicate that two of four epitopes detected in the standard antiviral response

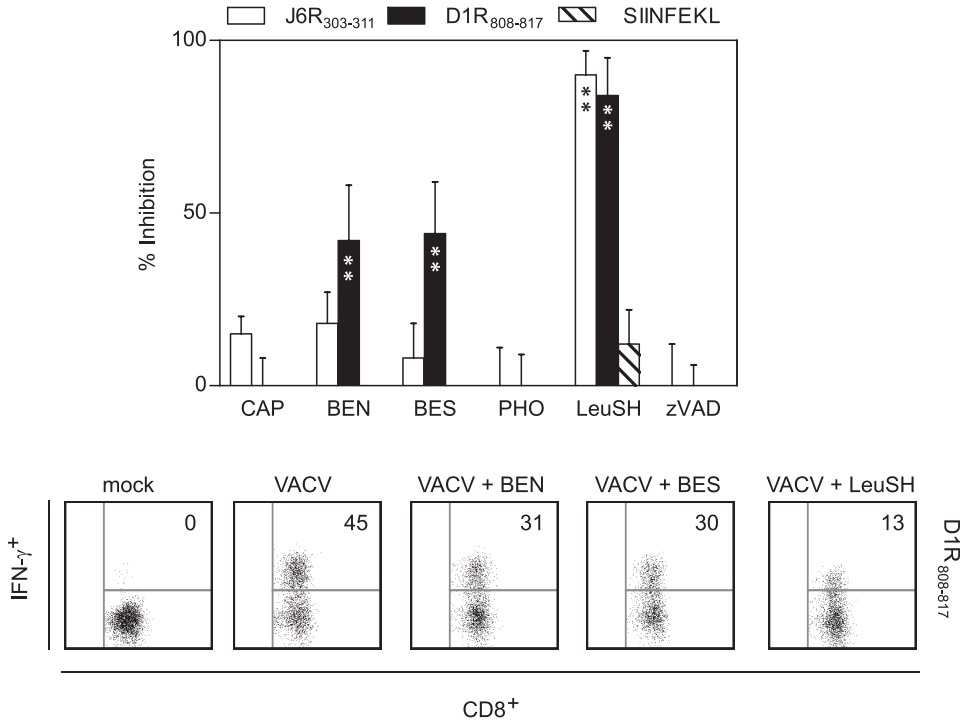

FIGURE 6. **Recognition of target cells infected in the presence of metalloproteinase subfamily inhibitors.** Cells infected as described in Fig. 4 were treated with captopril (*CAP*) (ACE-like metallopeptidase inhibitor), BEN (inhibits metallo-carboxypeptidases), BES (metallo-aminopeptidase inhibitor), phosphoramidon (*PHO*) (bacterial metalloendopeptidase inhibitor), LeuSH (mainly ERAP and other metallo-aminopeptidase inhibitor), or benzyloxycarbonyl-VAD (*zVAD*) (blocks caspases) as indicated, before the ICS assay. The figure is labeled as in Fig. 5. The percentage of specific inhibition was calculated as in Fig. 4. The data are means ± S.D. of three to four independent experiments. Significant *p* value, **, $p < 0.01$. Representative ICS assays with D1R(808–817)-specific $CD8^+$ T cell lines are depicted in the *bottom panels*. The percentages of IFN-γ-expressing $CD8^+$ T cells are indicated in each dot plot. *ACE*, angiotensin converting enzyme.

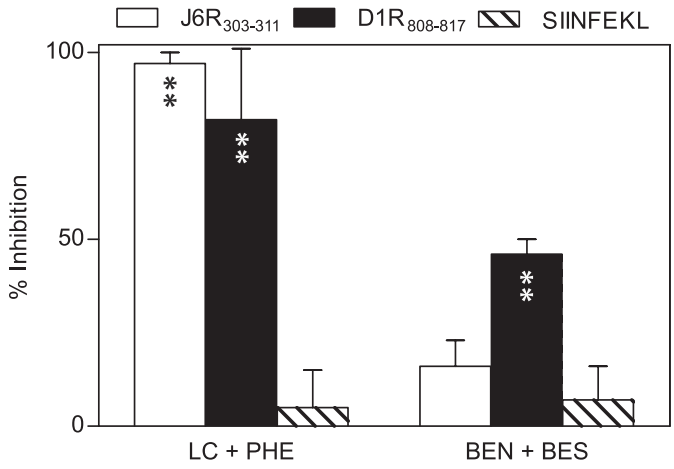

FIGURE 7. **Effect of combinations of inhibitors in the recognition of VACV-infected cells.** Cells infected as described in Fig. 4 were treated with the combination of either LC and PHE or BEN and BES, as indicated, before the ICS assay. The figure is labeled as in Fig. 5. The percentage of specific inhibition was calculated as in Fig. 4. The data are means ± S.D. of three or four independent experiments. Significant *p* value, **, $p < 0.01$. *ACE*, angiotensin converting enzyme.

from the H-2 class I double knock-out HLA-B*0702 transgenic mice (14) are presented by TAP-independent pathways as follows: the immunodominant D1R(808–817) epitope and the J6R(303–311) vaccinia peptide detected in human donors. Thus, TAP-independent HLA-B*0702 antigen presentation is sufficient to control vaccinia virus infection in the absence of a functional TAP complex. If these data are typical for all HLA class I molecules, this may help explain why individuals with unusable TAP complexes do not seem particularly susceptible to viral infections and may appear asymptomatic for much of their lives (reviewed in Ref. 5).

The sources of the two TAP-independent epitopes identified were the vaccinia J6R and D1R proteins. The J6R protein is a component of the viral RNA polymerase complex (35). The D1R protein is the large subunit of the viral mRNA capping enzyme (36) and is needed for early transcription termination (37). Presumably, the RNA polymerase carries the capping enzyme along as it transverses the template as a transcription elongation complex (38). Presentation of cytosolic proteins in cells lacking TAP has been previously reported (39, 40). This presentation of peptides could occur by passive diffusion (41), hydrophobic peptides with the ability to traverse membranes (42), or unidentified transport. Thus, either these VACV proteins or their respective ligands could be accessible to HLA-containing compartments with resident proteases for their TAP-independent HLA class I antigen processing and presentation.

Several proteases have been implicated in the processing of endogenously synthesized antigens independent of the classical proteasome pathway as follows: signal peptidase (43, 44); furin (45, 46); tripeptidyl peptidase II (47–49); lysosomal chloroquine-sensitive enzymes (50, 51); and caspases (52, 53). In this study, diverse proteolytic activities are required to generate the two HLA-B7-restricted epitopes studied. Our results using various protease inhibitors (summarized in Table 2) are consistent with the models depicted in Fig. 8. The block with LC indicates that the proteasome plays a role in the processing of the

TABLE 2
Summary of inhibition patterns

Epitope	BFA[a]	LC	PHE	BEN	BES	LeuSH	LC + PHE	BEN + BES
J6R(303–311)	+++[b]	+	+	−	−	+++	+++	−
D1R(807–817)	+++	−	++	+	+	+++	+++	+

[a] For specificity of different inhibitors see Table 1.
[b] −, +, ++, and +++ indicate % inhibition <20, 40–60, 61–80, and >81% respectively. All + inhibitions show significant *p* values ($p < 0.01$) *versus* controls without an inhibitor.

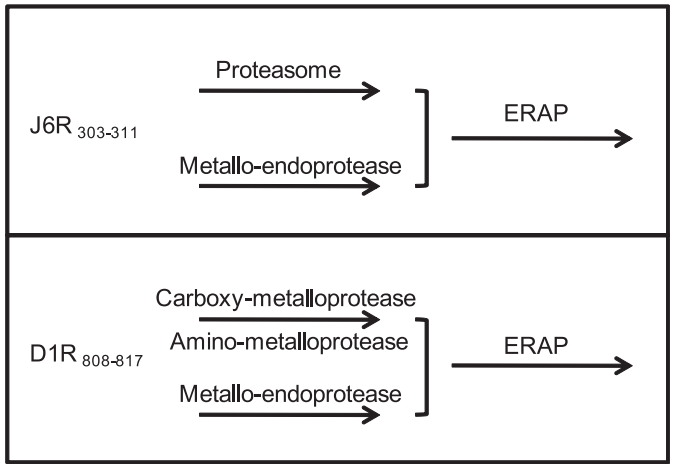

FIGURE 8. **Diversity of proteases and processing pathways involved in J6R(303–311) or D1R(808–817) epitope presentation.** The models show the components involved in each of the proposed pathways for the J6R(303–311) (*upper panel*) or D1R(808–817) (*lower panel*) epitopes. The role of proteases is deduced from the sensitivity of respective $CD8^+$ T cells to the various inhibitors (see Table 2).

J6R(303–311) epitope but not the D1R(808–817) viral ligand. The presentation of both vaccinia peptides was dependent on PHE-sensitive proteases, indicating that metalloprotease activity is required to process these epitopes. The LeuSH inhibitor impaired antigen recognition of target cells by both D1R(808–817) and J6R(303–311)-specific $CD8^+$ T cells. Because the pan-specific metalloprotease inhibitor PHE and the inhibitor of general aminoprotease activities BES did not block or had very little effect on ERAP (54, 55), the inhibition of D1R(808–817)- or J6R(303–311)-specific recognition requires both ERAP and other similar metalloproteases. In addition, the partial but selective impaired recognition of VACV-infected cells by D1R(808–817)-specific T cells in the presence of BEN or BES, which was not increased when both drugs were added together, implies that amino- (different from ERAP) and carboxy-metallopeptidases contribute sequentially to D1R(808–817) antigen processing. By contrast, the higher inhibition detected in the presence of LC and PHE *versus* single inhibitors demonstrated that the antigen processing of the J6R(303–311) epitope requires proteasomes and metallopeptidases independently.

Last, the recognition of VACV-infected cells by J6R(303–311)-specific $CD8^+$ T cells was partially blocked by PHE but not by the inhibitors of metalloproteases used in this study. Because phosphoramidon very efficiently inhibits bacterial metalloendopeptidases but does not block multiple higher vertebrate metallo-endoproteases, the most likely explanation for PHE-specific inhibition of J6R(303–311) recognition is that some mammalian metalloendopeptidases that are not blocked by phosphoramidon are involved in the antigen processing of this viral ligand. Similarly, there was higher inhibition of the antigen recognition of the D1R(808–817) epitope with PHE than with the combination of BEN and BES, suggesting additional metallo-endoprotease activity. More than 100 different well characterized higher vertebrate metalloendopeptidases are resistant to this reagent (33), and drugs that collectively and specifically block the endoproteolytic activity of this group of enzymes have not been described. Therefore, positive identification of the peptidase involved in the processing of these TAP-independent vaccinia epitopes awaits further characterization.

In summary, the J6R(303–311) product appears to be processed in parallel either by proteasomes or by metalloendopeptidases independently. Later, ERAP trims the final epitope. The processing of the D1R(808–817) epitope is more complex, involving a branched pathway in which metallo-endoproteases and a sequential cleavage of both metalloaminoprotease and metallocarboxyproteases are required to generate this epitope. As preceding epitope J6R(303–311), the trimming by ERAP generates the final D1R(808–817) epitope.

Metallopeptidases are among the hydrolases in which the nucleophilic attack on a peptide bond is mediated by a water molecule previously activated by a divalent metal cation (33). These proteases are allocated to 14 clans and subdivided into 62 families (MEROPS data base) (56). Based on their respective cleavage mechanism, these enzymes can be grouped into aminopeptidases, endopeptidases, carboxypeptidases, and carboxy-dipeptidases, among others (reviewed in Ref. 33). The ER resident amino-metallopeptidase ERAP plays an essential role in the trimming of different MHC class I ligands (3, 54, 57). In our study, we found that an ERAP-specific inhibitor blocks antigen presentation of both TAP-independent vaccinia ligands studied. Other amino-metallopeptidases also travel by the secretory pathway to their destination organelle or to the extracellular medium (33) and could be responsible for the BES-mediated inhibition observed in the antigen processing of the D1R(808–817) epitope. Also, carboxy-metallopeptidases abound in the secretory pathway and in vesicular compartments accessible to HLA class I molecules (33). Currently, no individual carboxypeptidases have been implicated in antigen processing in the vesicular pathway, but indirect evidence has been reported in two cases. First, the proteolytic action of furin in the secretory pathway is required to generate the antigenic CMV pp89 epitope located in the sHBe chimera (46). After this cleavage, nine C-terminal residues must be trimmed from the precursor peptide to generate the optimal 9pp89 epitope, and thus carboxypeptidases may be involved. Second, various signal sequence-derived peptides generated by signal peptidase complexes have C-terminally extended residues compared with the optimal HLA-bound epitope isolated (58–60); thus, carboxypeptidases may be involved in antigen processing of these

epitopes. This study directly implicates carboxy-metallopeptidases in the antigen processing of the vaccinia D1R(808–817) epitope. Finally, unknown metalloendopeptidases are involved in the processing of an HIV-1 epitope in a sequential TAP-dependent pathway that also implicates cleavage by proteasomes (15). In our study, proteasomes and uncharacterized metalloendopeptidases were found to be involved in J6R(303–311) cleavage in two different antigen-processing pathways.

In conclusion, the results reported here highlight the diversity of proteases involved in antigen recognition, and they uncover the complexity of antigen-processing pathways.

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Supplemental Table 1

VACV infection of the RMA cell line in the presence of various protease inhibitors

Condition [a]	Fluorescence Index ± SD [b]
Mock	1 ± 2
No inhibitor	27 ± 8
LC	28 ± 8
LEU	22 ± 5
PEP	23 ± 5
PHE	24 ± 9
E64	ND
PUR	ND
CAP	30 ± 10
BEN	28 ± 9
BES	ND
PHO	32 ± 7
LeuSH	34 ± 9
zVAD	27 ± 9

[a] RMA target cells infected for 16 hr with VACV at a multiplicity of infection of 40 plaque-forming units/cell were treated with the indicated inhibitors. A mock infected control was included as a negative control. The cells were stained with the Omnitope antiserum-FITC that recognizes VACV purified virions. Samples were analyzed by FACS.

[b] The results, calculated as fluorescence index ± SD, are means of 4 independent experiments. The fluorescence index was calculated as the ratio of mean channel fluorescence of the sample to that of the control incubated without VACV. All VACV-infected conditions (with and without inhibitors) show significant P values ($P < 0.01$) versus mock infected controls. By contrast, all inhibitor conditions show non significant P values versus VACV-infected control without an inhibitor. ND, not done.

Supplemental Table 2

Summary of HLA-B*0702 synthetic peptides from vaccinia virus

Ligand	Sequence	Recognition
A34R $_{82-90}$	LPRPDTRHL	Tg mice [a]
B ORF A $_{63-72}$	SPRLDTTYPL	
B ORF E $_{77-85}$	IPRHIFYQL	
B18R $_{305-313}$	RPADSITYL	
B19R $_{247-257}$	IPSQDHRFKL	
C1L $_{97-106}$	KPKPAVRFAI	Human [b]
D ORF H $_{50-58}$	HPRIFYQSL	
D1R $_{686-694}$	HPRHYATVM	Human
D1R $_{808-817}$	RPSTRNFFEL	Tg mice
D8L $_{148-156}$	APFDSVFYL	
E9L $_{623-631}$	IPRLLRTFL	
F4L $_{6-14}$	APNPNRFVI	Human
F5L $_{183-192}$	NPYPTESLAL	
F9L $_{129-137}$	APPGQHMLL	
H6R $_{245-254}$	LPSPKKLIAL	
I6L $_{257-266}$	FPTPKTFTPL	
J2R $_{116-124}$	KPFNNILNL	Tg mice
J3R $_{70-78}$	APGTHIRYL	
J3R $_{201-210}$	APSYSAEMRL	
J6R $_{303-311}$	MPAYIRNTL	Human
M1L $_{58-67}$	EPSGNNYHIL	

[a] Epitopes identified in HLA-B*0702 transgenic mice immunized with VACV (20).

[b] Epitopes identified in human donor vaccines (21;22).

EXPERT REVIEWS

Vaccination and the TAP-independent antigen processing pathways

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The cytotoxic $CD8^+$ T lymphocyte-mediated cellular response is important for the elimination of virus-infected cells and requires the prior recognition of short viral peptide antigens previously translocated to the endoplasmic reticulum by the transporter associated with antigen processing (TAP). However, individuals with nonfunctional TAP complexes or infected cells with TAP molecules blocked by specific viral proteins, such as the cowpoxvirus, a component of the first source of early empirical vaccination against smallpox, are still able to present several HLA class I ligands generated by the TAP-independent antigen processing pathways to specific cytotoxic $CD8^+$ T lymphocytes. Currently, bioterrorism and emerging infectious diseases have renewed interest in poxviruses. Recent works that have identified HLA class I ligands and epitopes in virus-infected TAP-deficient cells have implications for the study of both the effectiveness of early empirical vaccination and the analysis of HLA class I antigen processing in TAP-deficient subjects.

KEYWORDS: antigen processing • major histocompatibility complex • metalloprotease • poxviruses • protease inhibitors • proteasome • proteolytic enzymes • T cell • transporter associated with antigen processing • vaccine

Historical view of smallpox vaccination

Among the multiple pandemic contagious diseases that have plagued the human race since time immemorial, most likely none of them has had such a universal and continual impact on the human population as smallpox. Since its emergence, most likely in the first irrigated agricultural settlements, it is estimated that variola virus has been responsible for nearly one billion deaths and severely altered the course of history in different times, even contributing to the decline of several human civilizations [1]. Early efforts to control this pandemic disease by inoculation with smallpox pus or scabs have been historically documented from ancient times in several oriental cultures, and the technique of variolation gradually spread from these cultures to south-western Asia, the Ottoman Empire and later to Europe and the various European colonies [2].

Although in general, variolation was a relatively effective and preventive measure against smallpox, unfortunately, some subjects inoculated with scabs that contained infectious variola virus died and others transmitted the disease to the neighboring susceptible population. The low vulnerability of milkmaids to smallpox was widely known in several rural areas of various European countries. This observation was used by Edward Jenner and other English and Dutch physicians and even some concerned farmers to inoculate volunteers with fluid extracted from pustules on the hands of cowpox-infected milkmaids[2]. Later, the use of arm-to-arm pustule fluid as a means of vaccination was quickly accepted in Europe (including the Ottoman Empire), overseas European colonies and the newly independent USA, leading to the era of prophylactic vaccines.

These old smallpox vaccines and others obtained later in different European countries when the Jenner technique was accepted worldwide were randomly mixed during the nineteenth century to propagate complex mixtures of vaccine viruses similar to quasispecies [3]. Two centuries after this early empirical cross-protective vaccination with cowpox and horsepox virus, health efforts culminated with a massive worldwide vaccination program coordinated by the WHO that eradicated

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smallpox [4]. In this global program, vaccinia virus (VACV), another poxvirus that is related to smallpox, horsepox and cowpox [5], was used as the active principle. Currently, the origin of VACV remains uncertain. A reasonable explanation is that it is derived from either horsepox [6,3] or cowpox [7,8], and cowpox could be the most ancient of all poxviruses [9]. Vaccination induces a strong humoral response, leading to viral clearance, and the role of cellular responses in this cross-protection is well documented [10,11].

Orthopoxviruses & vaccines

Orthopoxviruses have large and complex virions that are visible by light microscopy. Their genome is a double-stranded DNA molecule of 130–375 kbp that varies between genera. Thus, variola major has a genome of 190 kbp and encodes approximately 200 proteins, whereas the VACV and cowpox genomes are somewhat larger, with a size of 200 and 224 kbp encoding approximately 220 and 240 proteins, respectively [5]. Poxvirus proteomes include numerous immunomodulatory proteins that disrupt the host immune response (reviewed in [12,13], including secreted proteins directed toward altering the function of chemokines, complement, cytokines and interferons and different intracellular immunomodulators that could disrupt the antiviral effects of interferons, apoptosis, host gene transcription and innate immune signaling. Particularly, orthopoxvirus immune evasion mechanisms involve blocking the trafficking of assembled MHC class I proteins in the endoplasmic reticulum (ER) to the plasma membrane by a KDEL-mediated retention pathway [14] or decreasing MHC class II expression on antigen-presenting cells after infection [15]. Many of these immune evasion strategies are common among different members of the poxvirus family, but some are specific to individual poxviruses.

Of particular interest is the cowpox protein CPXV12, which has no homologs in VACV or variola viruses. CPXV12 interferes with the human leukocyte antigen (HLA) class I–peptide complex folding by inhibiting peptide translocation by the transporter associated with antigen processing (TAP) [16]. These TAP heterodimers play a pivotal role in the classical antigen presentation pathway. The inhibition of these heterodimers prevents the translocation of most viral epitopes, which are mainly generated by the proteolytic degradation of viral proteins by the proteasome and other cytosol proteases, to the ER. There, these epitopes must be folded into HLA class I structures prior to their specific recognition on the surface of the infected cells by $CD8^+$ cytolytic T lymphocytes (CTLs) [17].

TAP-independent responses & TAP-deficient individuals

Thus, the TAP-independent antigen processing and presentation pathways must be important to generate the cross-reactive HLA class I cowpox epitopes that could be subsequently protective in subjects exposed to variola virus and contributed with other immune system layers to the successful early empirical vaccination against the smallpox pandemic. In addition, mutations in the TAP genes that generate non-functional TAP complexes have been described in both humans [18] and mice [19]. The few dozens of human patients with this HLA class I deficiency show a reduced functional $CD8^+$ T-cell population, but they may appear asymptomatic for long periods of their lives. TAP-deficient patients do not seem particularly susceptible to neoplasms or viral infections (largely and highly dependent on the T-cell immune responses) but show susceptibility to some chronic respiratory bacterial infections [18]. Therefore, the immune system of these TAP-deficient individuals must be reasonably competent, and NK cells, antibodies from B cells, $CD8^+$ $\gamma\delta$T cells and the decreased cytotoxic $CD8^+$ $\alpha\beta$ T subpopulation that is specific for TAP-independent antigens may all generate competent immune defenses that protect against severe viral infections in these individuals. These data reinforce the relevance of the TAP-independent antigen processing and presentation pathways (in addition to other immune mechanisms such as humoral responses [20]) in the effective control of viral pathogens.

Immunoproteomics of TAP-independent HLA class I ligands from orthopoxvirus

In recent years, various studies in either humanized HLA-transgenic mouse models or vaccinated humans have allowed the identification of more than 170 or 120 epitopes presented by different HLA class I or class II molecules, respectively, from multiple VACV proteins (reviewed in [21,22] and Immunoepitope Database) in TAP-sufficient models. In addition, several recent studies carried out in our laboratory have tried to further investigate two issues: TAP blocking by the cowpox virus and human TAP deficiency using the same viral model but with two different experimental approaches. First, a TAP-deficient human cell line that expresses four common HLA class I molecules on its surface was used to identify TAP-independent viral ligands that were simultaneously presented in the same infected TAP-deficient cells. The HLA-bound peptide pools were sequentially isolated from large numbers of TAP-deficient VACV-infected cells using specific antibodies. Mass spectrometry analysis identified 11 viral ligands bound to the four HLA class I molecules expressed by the TAP-deficient cells (summary in Table 1). Although most ligands were restricted by a single HLA class I allele, VACV ligands $K2L_{16–30}$ and $C11R_{101–110}$ were found in association with HLA class I molecules HLA–Cw1 and either HLA-B27 or HLA-B51, respectively. Therefore, in the same infected TAP-deficient cells, 13 different natural peptide–HLA class I complexes were formed simultaneously [23]. A similar number of ligands (3 or 4) associated to HLA class I molecules previously described to have high (HLA-B27) [24], low (HLA-A2) [25,26] or unknown (HLA-B51 and –Cw1) TAP dependency were identified. In a more recent study, the $D8L_{112–119}$ viral ligand, previously described to be presented on the classical HLA-Cw1 class I allele, was also physiologically presented by the non-classical HLA-E class I molecule in TAP-deficient cells [27]. In summary, these data indicate that 14 different natural peptide–HLA class I complexes of five HLA class I molecules (HLA-A2, -B27, -B51, -Cw1, and -E) were simultaneously presented in the same infected TAP-deficient cells (Table 1).

Table 1. Summary of HLA molecules bound by VACV TAP-independent ligands.

Ligand	Sequence	HLA restriction	Detection method	$CD8^+$ T-cell response	Ref.
A17L $_{9–17}$	MLDDFSAGA	-A2	IP†	Yes	[23]
A10L $_{614–623}$	SPEGEETII	-A2	IP	Yes	[23]
A10L $_{688–696}$	ILDRIITNA	-A2	IP	Yes	[23]
A10L $_{867–876}$	SRGYFEHMKK	-B27	IP	ND‡	[23]
B8R $_{53–59}$	WQTMYTN	-B27	IP	ND	[23]
K2L $_{16–30}$	YRLQGFTNAGIVAYK	-B27, -Cw1	IP	ND	[23]
D5R $_{148–157}$	IAMKRTLLEL	-B51	IP	ND	[23]
A50R $_{294–301}$	LPFGSLGI	-B51	IP	ND	[23]
C11R $_{101–110}$	IPSPGIMLV	-B51, -Cw1	IP	ND	[23]
A17L $_{9–25}$	MLDDFSAGAGVLDKDL	-Cw1	IP	ND	[23]
D8L $_{112–119}$	DGLIISI	-Cw1, -E	IP	Yes (in HLA-E)	[23,27]
D1R $_{808–817}$	RPSTRNFFEL	-B7	T-cell line†	Yes	[28]
J6R $_{303–311}$	MPAYIRNTL	-B7	T-cell line	Yes	[28]

†IP: sequential immunoprecipitation with specific antibodies; T-cell line, TAP-independent HLA-B7-restricted polyclonal VACV-polyspecific $CD8^+$ T-cell line.
‡ND: not determined.

Antiviral T-cell responses in HLA class I-transgenic mice models

To quantify the TAP-independent immune response that is conserved among orthopoxviruses, a TAP-independent polyclonal VACV-polyspecific $CD8^+$ T-cell line from vaccinated HLA-B7 transgenic mice was generated [28]. Two of the seven peptides previously reported as HLA-B7 epitopes were identified from the HLA-B7 transgenic mice ($A34R_{82–90}$, $D1R_{808–817}$ and $J2R_{116–124}$) [29] or the human vaccines ($AC1L_{97–106}$, $D1R_{686–694}$, $F4L_{6–14}$ and $J6R_{303–311}$) [30,31] and were recognized by the TAP-independent CTL cell line. Thus, these peptides were presented by TAP-independent pathways (Table 1). Notably, the $D1R_{808–817}$ peptide identified as a TAP-independent peptide [28] was the immunodominant epitope in the standard antiviral response from these H-2 class I double-knockout HLA-B7-transgenic mice. Thus, TAP-independent HLA-B7 antigen presentation could contribute in controlling VACV infection in the absence of a functional TAP complex. If the data obtained with the HLA-B7 allele were representative of most HLA class I molecules, this may help to explain why individuals with nonfunctional TAP proteins do not seem to be particularly susceptible to viral infections and may appear asymptomatic for much of their lives (reviewed in [18]).

TAP-independent ligands & hydrophobicity

Although TAP-independent viral epitopes are known (reviewed in [25,32,33]), no methodical studies on TAP-independent antigen presentation with a single virus and different HLA molecules have been reported, with the exception of Epstein–Barr virus (EBV). Different studies (reviewed in [34]) have shown that CTLs from several donors recognize eight EBV epitopes from only three different viral proteins and are restricted by four HLA class I molecules in TAP-deficient cells. These epitopes are always located in the hydrophobic regions of the respective EBV proteins, and they are highly hydrophobic, unlike the TAP-dependent EBV epitopes (Figure 1). Thus, the global picture of these data with regard to EBV could suggest a limited TAP-independent antigen-processing

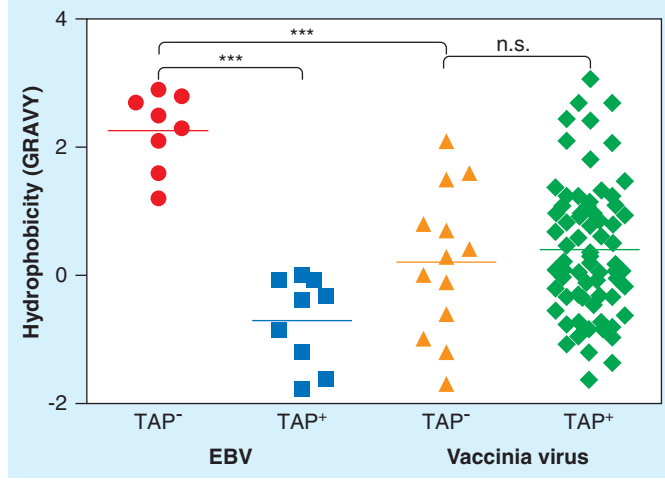

Figure 1. TAP-independent ligands/epitopes and their hydrophobicity are shown. Comparison of hydrophobicity measured on the grand average of hydropathicity (GRAVY) scale (ProtParam tool, ExPASy Proteomics Server [102]) of 8 TAP-independent (circles) and 9 TAP-dependent (squares) epitopes from EBV [34] versus 13 TAP-independent ligands/epitopes (triangles) [23,27,28] and 79 TAP-dependent epitopes (diamonds) [31,35] from VACV. The respective means are indicated as lines. ***Significant p-values ($p < 0.001$).

Table 2. Conservation ofTAP-independent viral HLA ligands in several orthopoxviruses.

Poxvirus†	$A17L_{9–17}$ (HLA-A2)	$A10L_{614–623}$ (HLA-A2)	$A10L_{688–696}$ (HLA-A2)	$D1R_{808–817}$ (HLA-B7)	$J6R_{303–311}$ (HLA-B7)	$A10L_{867–876}$ (HLA-B27)	$B8R_{53–59}$ (HLA-B27)
VACV	MLDDFSAGA	SPEGEETII	ILDRIITNA	RPSTRNFFEL	MPAYIRNTL	SRGYFEHMKK	WQTMYTN
Variola major	_ _ _ _ _ _ _ _ _	_ _ _ _ _ _ _ _ _	_ _ _ _ _ _ _ _ _	_ _ _ _ _ _ _ _ _	_ _ _ _ _ _ _ _ _	_ _ _ _ _ _ _ _ _	_ _ _ _ _ _ _ _ _
Variola minor	_ _ _ _ _ _ _ _ _	_ _ _ _ _ _ _ _ _	_ _ _ _ _ _ _ _ _	_ _ _ _ _ _ _ _ _	_ _ _ _ _ _ _ _ _	_ _ _ _ _ _ _ _ _	_ _ _ _ _ _ _ _ _
Cowpox	_ _ _ _ _ _ _ _ _	_ _ _ _ _ _ _ _ _	_ _ _ _ _ _ _ _ _	_ _ _ _ _ _ _ _ _	_ _ _ _ _ _ _ _ _	_ _ _ _ _ _ _ _ _	_ _ _ _ _ _ _ _ _
Poxvirus†	**$K2L_{16–30}$ (HLA-B27, -Cw1)**	**$D5R_{148–157}$ (HLA-B51)**	**$A50R_{294–301}$ (HLA-B51)**	**$C11R_{101–110}$ (HLA-B51, -Cw1)**	**$A17L_{9–25}$ (HLA-Cw1)**	**$D8L_{112–119}$ (HLA-Cw1, -E)**	
VACV	YRLQGFTNAGIVAYK	IAMKRTLLEL	LPFGSLGI	IPSPGIMLV	MLDDFSAGAGVLDKDL	DGLIIISI	
Variola major	_ _ _ _ _ _ _ _ _	_ _ _ _ _ _ _ _ _	_ _ _ _ _ _ _ _ _	_ _ _ _ _ V _ _ _	_ _ _ _ _ _ _ _ _	_ _ _ _ _ _ _ _ _	
Variola minor	_ _ _ _ _ _ _ _ _	_ _ _ _ _ _ _ _ _	_ _ _ _ _ _ _ _ _	_ _ _ _ _ V _ _ _	_ _ _ _ _ _ _ _ _	_ _ _ _ _ _ _ _ _	
Cowpox	_ _ _ _ _ _ _ _ _	_ _ _ _ _ _ _ _ _	_ _ _ _ _ _ _ _ _	_ _ _ _ _ V _ _ _	_ _ _ _ _ _ _ _ _	_ _ _ _ _ _ _ _ _	

†The sequences used were obtained from the NCBI database [103].

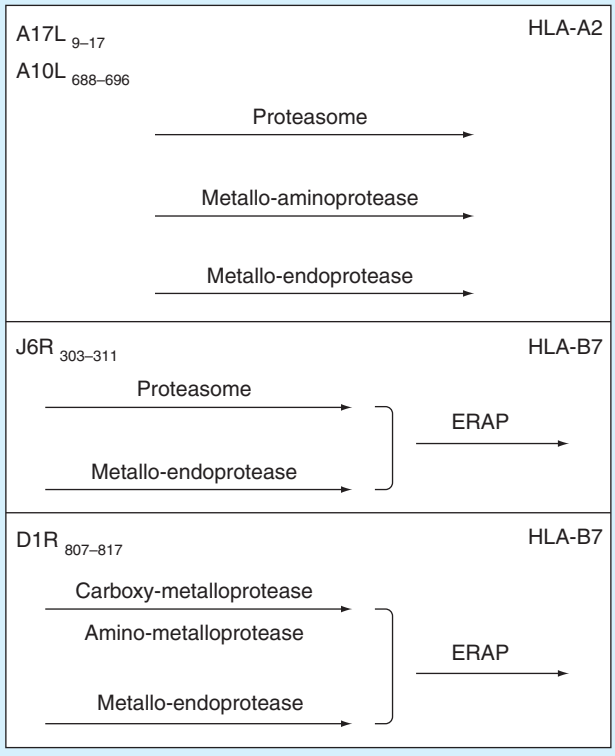

Figure 2. Diversity of proteases and processing pathways involved in TAP-independent VACV epitope presentation are illustrated. The models show the components involved in each of the proposed pathways for both HLA-A2-restricted ($A17L_{9–17}$ and $A10L_{688–696}$ [upper panel]) and HLA-B7-restricted ($J6R_{303–311}$ [middle panel] or $D1R_{808–817}$ [lower panel]) epitopes. The role of different proteases was deduced from the sensitivity of the respective $CD8^+$ T cells to the various protease inhibitors (see [23,28]).

ability focused on the hydrophobic regions of some proteins independently of the large genomic size of this herpesvirus (approximately 200 Kbp, similar to poxviruses).

In contrast, TAP-independent antigen processing in TAP-negative cell backgrounds generates 13 ligands from 10 unrelated VACV proteins (expressed in the three gene expression temporality clusters of the viral lifecycle) presented by six different HLA class I classical and non-classical molecules (Table 1). No obvious protein patterns, such as gene expression, function, viral lifecycle, localization and domains or ligand characteristics, such as position and sequence (data not shown), including their specific hydrophobicity (shown in Figure 1), were found. Therefore, hydrophobicity is not a necessary condition for the TAP-independent presentation of ligands/epitopes for all viruses. Furthermore, VACV TAP-independent antigen processing appears to be less restricted than EBV TAP-independent presentation.

Expert commentary

Only the HLA class I epitopes conserved between the vaccine virus (cowpox or VACV) and the pathogenic variola virus were responsible for the cross-reactive protection in individuals exposed to variola virus. Variola virus shows 72 and 82% amino acid homology with cowpox and VACV proteomes, respectively (Poxvirus Bioinformatics Resource Center [101]). As the amino acid differences are not equally distributed in the viral proteome, the conservation analysis of the 79 TAP^+ VACV epitopes previously described [31,35] between both poxviruses shows that 60% of these epitopes are conserved in the variola proteome. When a similar analysis was performed with the sequences of 13 TAP-independent VACV ligands that were previously identified [23,27,28], the ligands were found to be highly conserved among orthopoxviruses (Table 2). Only a minor substitution at the P7 position of one of the 13 ligands (sequence $C11R_{101–110}$) was found and thus 12 ligands (92%) are fully conserved in variola major, variola minor and cowpox viruses. These data show

that TAP-independent VACV ligands are more conserved than TAP-dependent epitopes between pathologic and immunogenic poxviruses, which could explain the effectiveness of early empirical vaccination with cowpox virus against smallpox disease.

So far, different proteases have been implicated in the processing of endogenously synthesized antigens independent of the classical proteasome pathway: signal peptidase [36,37], furin [38,39], tripeptidyl peptidase II [40,42], lysosomal chloroquine-sensitive enzymes [43–45] and caspases [46,47]. In our recent studies [23,28], a systematic analysis of the antigen processing pathways involved in the endogenous generation of the HLA-A2 and HLA-B7 TAP-independent viral epitopes generated in VACV-infected cells was performed. This analysis was performed using polyclonal $CD8^+$ T cell lines that were monospecific for each viral epitope produced from the HLA transgenic mouse models in the presence of diverse protease inhibitors in VACV-infected TAP-proficient cells. As shown in Figure 2, in normal cells, the presentation of four HLA-A2 and HLA-B7 epitopes occurs via complex pathways involving antigen processing by the proteasome and/or by diverse subsets of metalloproteinases (amino-, carboxy-, and endoproteases) acting in parallel or sequentially. These data support the fact that peptides recognized by the antiviral cellular immune response are supplied by different cellular proteolytic systems, thereby contributing and facilitating immunosurveillance.

Five-year view

In summary, the study of the HLA class I-mediated response in VACV-infected TAP-deficient cells results in a new and intricate picture of the TAP-independent antigen processing pathways, which could explain both the effectiveness of early empirical vaccination with cowpox virus against smallpox and why TAP-deficient individuals live for long periods without enhanced susceptibility to viral infections. In addition, the existence of these multiple TAP-independent VACV ligands generated by multiple antigen processing and presentation routes, which operate in the absence of the TAP-dependent classical pathway, suggests that these pathways could be a secondary but extended and relevant mechanism in addition to the multiple components of immune protection against viral infection, but this hypothesis requires further investigation including:

- Immunoproteomics analysis of human cowpox-infected cells;
- Analysis and quantification of the T-cell immune responses using cowpox-vaccinated HLA class I transgenic mice models;
- Identification of proteases that may contribute to the processing of TAP-independent HLA-class I epitopes from cowpox-infected cells;
- Role of viral TAP-independent HLA-class I ligands in NK cell recognition.

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Key issues

- The TAP delivers the viral proteolytic products generated by the proteasome in the cytosol to the ER lumen that are subsequently recognized by cytotoxic T lymphocytes (CTL).
- Individuals with mutations in the TAP gene that generate non-functional TAP complexes do not seem particularly susceptible to viral infections. Thus, the reduced CTL subpopulation that is specific for TAP-independent antigens may contribute to immune defenses that protect against severe viral infections in these individuals.
- The eradication of smallpox, a disease caused by variola major virus, was made possible by early empirical, cross-protective vaccination with both cow and horse orthopoxvirus.
- Cowpox virus specifically inhibits TAP-dependent peptide translocation; therefore, TAP-independent epitopes conserved between variola and this virus probably contributed to the initial cross-protection.
- Using mass spectrometry to analyze complex HLA-bound peptide pools isolated from large numbers of TAP-deficient, multiple viral ligands naturally presented by different HLA-A, -B, -C and -E class I molecules and conserved among the *Orthopoxviridae* family were identified.
- Two of four epitopes (including the immunodominant epitope) detected in the standard antiviral response from the H-2 class I double-knockout HLA-B*07-transgenic mice were presented by TAP-independent pathways. Thus, TAP-independent HLA-B*07 antigen presentation could be sufficient to control orthopoxvirus infection in the absence of a functional TAP complex.
- The existence of multiple TAP-independent orthopoxvirus ligands generated by multiple proteases in complex antigen processing and presentation routes, which operate in the absence of the TAP-dependent classical pathway, suggests that these pathways could be a secondary but extended and relevant mechanism of immune protection against viral infection, but this hypothesis requires further investigation *in vivo*.

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VII.-DISCUSIÓN

El presente estudio es el análisis más completo realizado hasta la fecha sobre los ligandos de HLA-I endógenos y virales generados en células deficientes en TAP y sobre vías de procesamiento implicadas en la generación de estos ligandos. Para llevar a cabo el estudio de estas vías se analizaron por espectrometría de masas los péptidos unidos a distintos HLAs de una misma línea celular deficiente en TAP infectada con un virus vaccinia recombinante que expresa la gp160 de HIV. Así, se identificaron 12 ligandos virales (11 derivados de proteínas del virus vaccinia y 1 de la gp160 de HIV) y 334 endógenos, presentados por cinco alelos de HLA distintos (HLA-A*02, -B*27, -B*51, -C*01 y –E) algunos de ellos de forma promiscua. Además, mediante una segunda aproximación se describieron otros dos epítopos virales independientes de TAP presentados por HLA-B*07. En todos los alelos clásicos analizados se observaron varios ligandos virales y varias decenas de endógenos, lo que refuerza la idea de la existencia de una elevada diversidad de ligandos en las vías independientes de TAP.

Varios estudios previos han evaluado parcialmente el peptidoma de células deficientes en TAP. En el primero de ellos identificaron 46 ligandos presentados por la molécula de MHC-I de ratón K^d que presentaban de media un mayor tamaño y unos motivos de anclaje más laxos que los de sus análogos generados en células con TAP (109). Suri et al. demostraron además que algunos de estos ligandos eran presentados en la superficie celular por lo que serían productos finales de la vía de procesamiento (109). En un segundo trabajo, Weinzierl et al. identificaron 52 ligandos de HLA-A*02 o HLA-B*51 en células deficientes en TAP (26) con tamaños canónicos. Posteriormente se publicaron otros dos trabajos, en los que se analizaban los ligandos presentados por una molécula de MHC-I no clásica, tanto en ratón (Qa-1b) (110) como en su equivalente humano (HLA-E) (111). Las características del repertorio peptídico de ambos estudios eran similares a las identificadas previamente en el peptidoma de K^d. Además, en ausencia de TAP el repertorio peptídico presentado por Qa-1b y HLA-E es mucho más variado que en las células con el transportador, en las que este MHC-I no clásico une principalmente péptidos derivados de la secuencias señal de las moléculas de MHC-I clásicas. Los 334 ligandos endógenos identificados en el presente trabajo mostraron también un mayor tamaño y unos motivos de anclaje más laxos para todos los alelos de HLA analizados. Por tanto, los datos presentados en esta memoria junto con la mayoría de los estudios similares indican que la ausencia de TAP enriquece el repertorio de HLA en péptidos largos, lo que es consistente con el papel del transportador en la selección de epítopos de menor longitud (148). Además, dadas las características de los ligandos y la similitud de las proteínas de las que derivan, las vías de procesamiento implicadas en la generación del peptidoma de las moléculas de MHC-I clásicas y no clásicas podrían ser las mismas.

El análisis de los ligandos endógenos identificados en la presente memoria reveló que sorprendentemente un tercio de los ligandos identificados procedían de solo 10 proteínas, lo que supone un elevado grado de degradación de estos sustratos o la existencia de un compartimento al que solo tienen acceso un número limitado de proteínas. También se identificaron regiones con una elevada densidad de ligandos unidos a los distintos HLAs. Estas regiones se han descrito anteriormente en algunas proteínas patogénicas, especialmente en HIV (149,150). Otra característica relevante de los péptidos endógenos identificados es que casi el 25% de ellos se encuentran ubicados en el extremo carboxilo de la proteína de la que proceden. Este tipo de péptidos se han identificado también unidos a otros alelos de MHC-I en células deficientes en TAP (109,110). En 1998 ya se describió una vía independiente de TAP capaz de generar ligandos de la región carboxilo terminal, pero a partir de proteínas solubles y de membrana presentes en la vía secretoria que son degradadas por enzimas proteolíticas como la furina (124,151).

En segundo lugar, la presente memoria se ha centrado en la caracterización de las vías de procesamiento independientes de TAP mediante dos aproximaciones distintas: el análisis bioinformático de los ligandos endógenos identificados por espectometría de masas y el estudio de la reexpresión de HLA-I en presencia de diferentes inhibidores de proteasas. Estas aproximaciones permiten obtener información complementaria, ya que el repertorio de ligandos independientes de TAP identificados procede del conjunto de la célula mientras que en el análisis de la expresión de HLA-I solo se evalúan los ligandos que se encuentran de manera estable en la superficie celular.

Los primeros trabajos con células deficientes en TAP sugerían que la peptidasa señal podría ser la principal responsable del procesamiento de los ligandos presentados por algunos alelos de HLA (13,14), pero la mayoría de los identificados posteriormente proceden de proteínas citosólicas y nucleares y no de secuencias señales (109-111), salvo en el trabajo de Weinzierl et al (26). En este último estudio solo analizaron los ligandos de HLA-A*02 y -B*51 comunes entre la línea celular TAP competente y la TAP deficiente, perdiéndose la información de todos aquellos ligandos generados solo por una de las líneas celulares o que se encuentren por debajo del límite de detección en una de ellas. De todos los ligandos endógenos identificados en esta memoria solo el 11% de los presentados por HLA-A*02 y el 3% de los unidos por HLA-B*27, -B*51 y -C*01 derivaron de secuencias señal, mientras que los resultados obtenidos mediante los ensayos de reexpresión de HLA-I indicaron que esta vía de procesamiento contribuiría a la formación del 50% de los complejos HLA-B*51/péptido presentados en la superficie celular y del 80% de los HLA-A*02/péptido. Una explicación plausible de esta discrepancia podría ser que los ligandos derivados de secuencias señal fuesen generados más eficientemente, ya que

todas las proteínas que presentan estas regiones deben ser procesadas por la SP, siendo por ello los responsables de la mayoría de la expresión en superficie de los alelos de HLA-I que pueden interaccionan con péptidos con residuos hidrofóbicos y neutros, como HLA-A*02 y -B*51. Aunque también podría haberse sobreestimado la contribución de esta vía de procesamiento si parte de la inhibición detectada en los ensayos de reexpresión de HLA-I en presencia del inhibidor de la SPP se debieran a una función alternativa de la peptidasa. En este sentido, recientemente se ha implicado a la SPP en la generación de un epítopo tumoral localizado en el extremo carboxilo de una proteína con dominio transmembrana, independientemente de la SP (119). Además, la SPP2a, un miembro de la familia SPP que se encuentra en los lisosomas/endosomas tardíos (152), participa en la proteolisis de la cadena invariante y es crítica para el desarrollo y la supervivencia de las células tanto B como dendríticas (153-155), por lo que podría afectar indirectamente a la expresión de MHC-I en superficie.

En un trabajo previo se describió que el complejo proteolítico implicado en la generación de la mayoría de los ligandos en las células con TAP, el proteasoma, es también el responsable de la mitad de los ligandos de HLA-A*02 y -B*51 en las células deficientes en TAP (26). Además en otro trabajo posterior al publicado con los datos de la presente memoria, en el que también analizaban los niveles de expresión de MHC-I en la superficie celular pero en células deficientes en TAP de origen murino, se ha identificado al proteasoma y a metaloproteasas como las principales proteasas responsables de la generación de ligandos independientemente de TAP (156). En células TAP-competentes se ha observado que la contribución del proteasoma a la expresión en la superficie celular del HLA es dependiente de alelo (157). Los resultados obtenidos en los ensayos de inhibición en las células deficientes en TAP mostraron una dependencia similar a la observada en las células normales, siendo HLA-B*27 el alelo menos afectado por los inhibidores de proteasoma. En un estudio previo en el que analizaban el repertorio peptídico de células TAP competentes en presencia de inhibidores del proteasoma se ha observado que la mayoría de los ligandos endógenos de HLA-B*27 procedían de proteínas básicas de pequeño tamaño (158). Un análisis similar de las proteínas de las que proceden los ligandos de HLA-B*27 identificados en la actual memoria mostró que la mayoría de las proteínas son básicas pero de mayor tamaño, mientras que en HLA-A*02 son ácidas y también de mayor tamaño. Por tanto, es posible que las vías que son capaces de generar ligandos de HLA en presencia de inhibidores del proteasoma no sean las mismas que actúan en ausencia de TAP. Se desconoce el método por el cual los péptidos procesados por el proteasoma podrían acceder a un compartimento en el que se localice el MHC-I, aunque se han propuesto diferentes mecanismos de transporte alternativo como la difusión

pasiva, el transporte a través del translocon Sec61, TAPL (122) u otro transportador no identificado (120).

En los datos expuestos en la presente memoria también se ha observado la contribución a la generación del repertorio peptídico de HLA-B*27 de una vía sensible a cloroquina (CQ) lo que implica que un porcentaje significativo de estas moléculas de HLA estarían accediendo a un compartimento endolisosomal donde unirían ligando, en consonancia con algunos estudios previos (159-161). Las vías sensibles a CQ suelen ser frecuentes en respuestas frente a proteínas virales exógenas y virus inactivados tanto *in vitro* (162,163) como *in vivo* (164). La mayoría de estas vías sensibles a CQ no se ven afectadas por la inhibición del transporte anterógrado desde el Golgi de la vía secretoria por la brefeldina (BFA), pero en algunos casos se ha descrito la implicación de los lisosomas (165) y de la autofagia en el procesamiento endógeno de algunos epítopos virales de MHC-I tanto en vías dependientes (131) como independientes de TAP (166). La expresión de HLA-B*27 en las células deficientes en TAP empleadas en la presente memoria fue dependiente de BFA, por lo que el origen de sus ligandos sería mayoritariamente endógeno e implicaría que aquellas proteínas que pudieran acceder al compartimento endolisosomal serían la fuente principal del ligandoma independiente de TAP presentado por HLA-B*27.

Muchas endoproteasas tienen preferencia por sustratos con uno o varios aminoácidos determinados en posiciones adyacentes al sitio de corte. Por ello, se valoró la existencia de patrones de corte en las posiciones adyacentes (P3, P2, P1, P1', P2' y P3') (Figura 1) a los sitios de escisión que generaron a los ligandos endógenos independientes de TAP presentados en la actual memoria. Este análisis puso de manifiesto la existencia de dos actividades endoproteolíticas diferentes, capaces de procesar una misma proteína, una con especificidad por los residuos K/R y otra por F/L en posición P1. Estos dos tipos de proteasas serían las responsables de más de la mitad de los ligandos identificados. En el resto de las posiciones evaluadas no se detectó ningún patrón de corte específico.

Los datos presentados hasta el momento, representados en la figura 2, evidencian la implicación diferencial de las distintas proteasas en la generación del repertorio peptídico generado en células deficientes en TAP, en función del alelo analizado. Esto puede deberse a los diversos requerimientos estructurales de los péptidos que une cada alelo, por ejemplo HLA-A*02 interacciona con péptidos hidrofóbicos y neutros similares a las regiones de secuencia señal, mientras que HLA-B*27 une péptidos básicos.

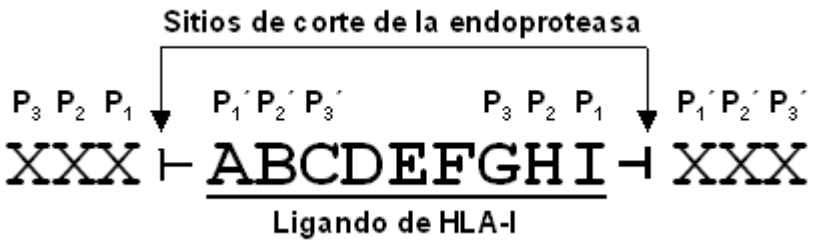

Figura 1.- Diagrama de los residuos adyacentes a los extremos amino y carboxilo de los ligandos de HLA.

Se muestran las posiciones adyacentes a los sitios de escisión evaluadas en la búsqueda de patrones de corte de las proteasas implicadas en la generación de los ligandos endógenos. La secuencia ABCDEFGHI representa un ligando de HLA-I cualquiera.

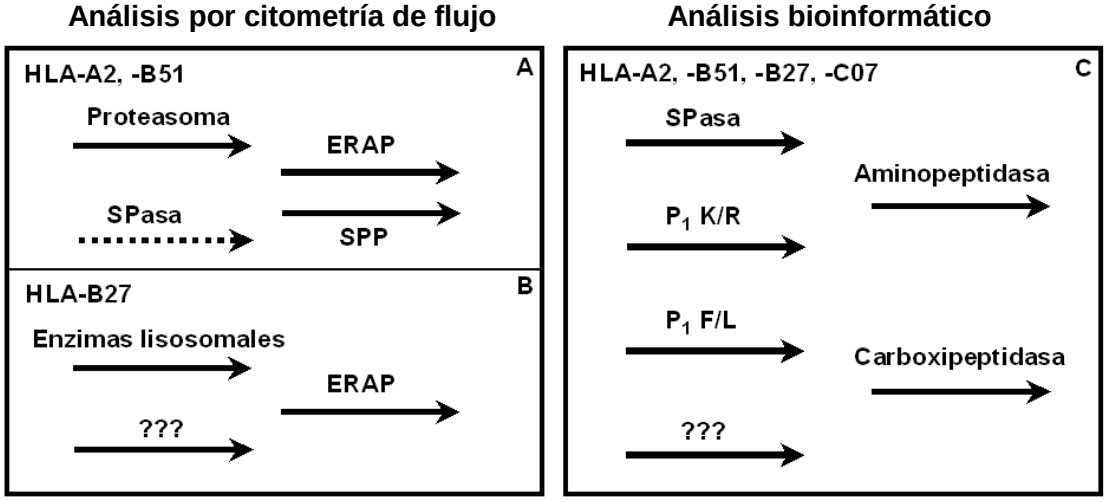

Figura 2.- Esquema de las vías de procesamiento implicadas en la generación de los ligandos endógenos.

Se muestra la diversidad de proteasas implicadas en el procesamiento de los ligandos endógenos presentados por los distintos alelos de HLA. La implicación de dichas proteasas se deduce de los ensayos de inhibición de la expresión de los distintos alelos de HLA en la superficie celular (A y B) o del análisis de las características de los distintos ligandos endógenos identificados por espectometría de masas de la misma línea celular (C). La implicación de la SPasa en el panel A es indirecta, por la inhibición de la expresión en presencia del inhibidor de la SPP.

El análisis de las secuencias de los ligandos endógenos de HLA-I descritos también sugiere la existencia de amino y carboxipeptidasas implicadas en su procesamiento, ya que casi el 20% de los ligandos presentaron extensiones en el extremo amino o/y en el carboxilo respecto al núcleo peptídico mínimo detectado. En los ensayos de reexpresión de los distintos alelos de HLA también se observó una actividad, inhibible por leucintiol, responsable del procesamiento de la mitad de los ligandos de HLA-A*02, -B*27 y -B*51 que podría deberse a ERAP o a otra aminopeptidasa sensible a este inhibidor. Mientras que por otra parte ACE o a alguna otra carboxipeptidasa aún no identificada podría ser la responsable de la actividad observada. En un trabajo reciente en el que se identificaron

12000 ligandos de HLA-I presentados por una línea celular con TAP también se obtuvo un porcentaje significativo de péptidos extendidos en el extremo amino (del 0.5-2% de los ligandos) y en el carboxilo (del 2-7%) dependiendo del alelo analizado (167). Que tanto en este trabajo como en los resultados obtenidos en la presente memoria se haya identificado un porcentaje significativo de ligandos de HLA con extensiones en el extremo carboxilo puede deberse a que estos ligandos se encuentran en baja concentración y, por tanto, solo se detectan con equipos de última generación o debido a que la mayoría de los ligandos más abundantes se encuentran ausentes en las células deficientes en TAP.

En el presente trabajo, así como en otros análisis inmunoproteómicos (110,111), se ha identificado un número importante de ligandos procesados independientemente de TAP que, al igual que el peptidoma de MHC-II, son péptidos anidados, con el mismo núcleo peptídico y distinta longitud. Es posible que las moléculas de MHC-I que recirculan desde la superficie celular sean capaces también de unir péptidos presentes en algún tipo de vesícula y que por ello, las proteínas de las que proceden los ligandos identificados en la presente memoria sean similares a las descritas en distintos tipos de estructuras vesiculares, especialmente a las de las vesículas secretorias de los neutrófilos (168-170). En otros estudios con células deficientes en TAP se han identificado diversos alelos de MHC-I unidos a la cadena invariante (171,172), lo que podría permitir su entrada en compartimentos endolisosomales donde habitualmente se unen los péptidos a las moléculas de MHC-II. En la línea celular empleada en esta memoria podría además potenciarse esta interacción con la cadena invariante dado que la célula carece de HLA-II, su ligando natural. Este papel de la cadena invariante, ya se ha descrito en presentación cruzada de antígenos exógenos en células dendríticas, tanto en presencia como en ausencia de TAP (173). También es posible que exista otro transportador que permita la entrada de péptidos en algún compartimento al que accedan las moléculas de MHC-I vacías o procedentes del reciclaje desde la membrana celular. En 2007 se identificó un segundo transportador de péptidos, el ABCB9 o TAPL (122), localizado en la membrana lisosomal de las células dendríticas y macrófagos, de tal manera que este u otro transportador podría ser relevante en la presentación antigénica en células deficientes en TAP. Esta última hipótesis parece menos probable ya que los resultados obtenidos en la actual memoria sugieren que es más factible que sea la proteína la que entre en el compartimento donde se encuentra el MHC-I en vez de los péptidos ya procesados, puesto que se identifican múltiples ligandos de una misma proteína y que incluso en algunos casos como en la miosina, estos ligandos se encuentran adyacentes en la secuencia de la proteína. En los últimos años se han publicado diversos trabajos en los que se observa que las vías de procesamiento responsables de la generación de ligandos de HLA-I y HLA-

II no son tan diferentes como se suponía en un principio (132,174,175). Por ejemplo, el repertorio peptídico de HLA-II se ve modificado en ausencia de TAP y de ERAP (175). Además se han identificado ligandos de HLA-II generados a partir de proteínas virales endógenas (176). En la generación de algunos de estos ligandos se ha observado que el proteasoma tiene un papel importante, mientras que TAP y LAMP-2 no tienen ninguna relevancia (177). Esta vía de procesamiento podría contribuir también a la generación de los ligandos de HLA-I en las células deficientes en TAP, por lo menos del repertorio presentado por los alelos A*02 y B*51 que se ve afectado por inhibidores del proteasoma.

Finalmente, la figura 3 representa el modelo global de las vías de procesamiento identificadas en la presente memoria, en el cual se pone de manifiesto una elevada complejidad de rutas y de proteasas implicadas en la generación de los ligandos endógenos. Además, hay que destacar que no todas las vías de procesamiento contribuyen por igual al repertorio peptídico presentado por cada uno de los distintos alelos de HLA analizados.

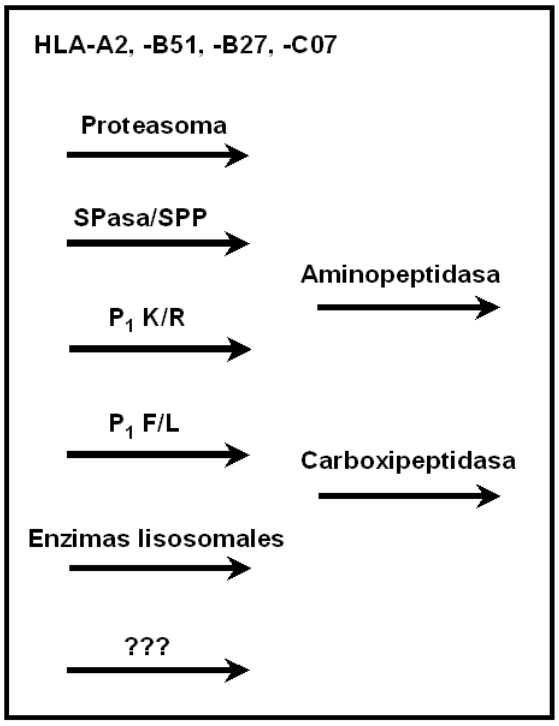

Figura 3.- Esquema de las vías de procesamiento identificadas en la generación de los ligandos endógenos en células deficientes en TAP.
Se muestra el resumen de todas las proteasas identificadas en el procesamiento de los ligandos endógenos en las células deficientes en TAP.

Un tercer aspecto analizado en la presente memoria fue el estudio de las vías de procesamiento implicadas en la generación de los ligandos virales independientes de TAP. (Figura 4). El proteasoma y metalo-proteasas, posiblemente con actividad endoproteolítica,

contribuyeron a la generación de la mayoría de estos epítopos mediante vías de procesamiento paralelas. La implicación del proteasoma podría venir dada por su contribución a la vía clásica de procesamiento antigénico o a vías alternativas independientes de TAP, tal y como se ha indicado previamente en la presente memoria y en un estudio previo (26). Además, los ensayos con los distintos inhibidores mostraron la implicación de una o varias metalo-endoproteasas en el procesamiento antigénico de estos epítopos. En la bibliografía existen datos sobre la capacidad de varias metalo-endoproteasas de generar péptidos del tamaño adecuado para ser presentados por MHC-I. En un trabajo previo en el que se realizaban estudios de inhibición similares a los aquí descritos, se involucraba a una metalo-endoproteasa en el procesamiento de un epítopo de VIH (178). Además, en los últimos años se han identificado dos metalo-endoproteasas, nardilisina y TOP, que son necesarias para complementar la actividad del proteasoma en el procesamiento por vías dependientes de TAP de varios epítopos de CTLs (4). Dada la especificidad de la nardilisina por residuos dibásicos, ausentes en las secuencias flanqueantes de los ligandos virales identificados, y que ambas enzimas tienen afinidad por sustratos de pequeño tamaño no parece probable que ninguna de estas peptidasas contribuyan a la generación de los epítopos descritos en la presente memoria. Además, la metalo-endoproteasa IDE ha sido implicada en el procesamiento de un epítopo tumoral por una vía independiente al proteasoma (3). Por todo ello sería interesante en un futuro evaluar la implicación de esta enzima en el procesamiento de los ligandos virales o intentar caracterizar las metalo-endoproteasas implicadas en la generación de los epítopos de vaccinia estudiados.

Además, la ERAP (o alguna otra proteasa inhibible por leucintiol) también contribuyó al procesamiento de los ligandos virales caracterizados. En la generación de los dos ligandos restringidos por HLA-B*07, esta aminopeptidasa no estaría actuando solo en la vía del proteasoma, sino que también sería necesaria para el recorte del extremo amino-terminal tras la actividad de otras metalopeptidasas (Figura 4, panel B y C).

El epítopo D1R (Figura 4C), además de ser procesado por una metalo-endoproteasa y por una aminopeptidasa sensible a leucintiol, también requirió de la actividad de una metalo-aminopeptidasa inhibible por bestatina (BES) y de otra carboxipeptidasa sensible al ácido bencilsuccínico. Dado que la BES a la concentración utilizada en los ensayos no sería capaz de inhibir la actividad de ERAP (74,179), sería otra aminopeptidasa diferente la que colaboraría en el recorte del extremo amino del péptido. En la bibliografía se han descrito otras dos metalo-aminopeptidasas, PSA (180) y LAP (55), implicadas en el procesamiento de algunos epítopos de CTLs. Dado que la respuesta de los CTLs frente a células infectadas con vaccinia no se ve afectada en presencia de puromicina, la PSA no

sería responsable del recorte. Además, la actividad de LAP se inhibe en presencia de leucintiol y de BES, al igual que la aminopeptidasa implicada en la generación del epítopo de D1R, por lo que esta proteasa u otra metalo-aminopeptidasa aún no identificada podría ser la responsable del procesamiento del extremo amino del epítopo D1R. En la generación de este epítopo también se observó la implicación de una metalo-carboxipeptidasa, pero dado que esta actividad no se vio afectada por el inhibidor de ACE, los datos indican que habría al menos una segunda enzima implicada en procesamiento antigénico capaz de recortar el extremo carboxilo de un péptido.

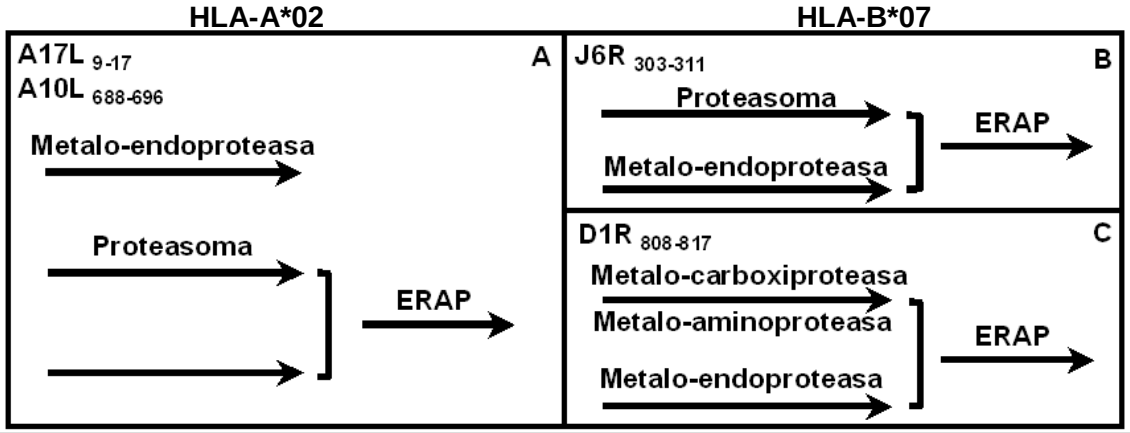

Figura 4.- Esquema de las vías de procesamiento implicadas en la generación de los epítopos virales de vaccinia.

Se muestra la diversidad de proteasas implicadas en el procesamiento de los distintos epítopos caracterizados: $A17L_{9-17}$ y $A10L_{688-696}$ en el panel A, $J6R_{303-311}$ en el B y $D1R_{808-817}$ en el C. La implicación de dichas proteasas se deduce de los ensayos de inhibición realizados con los CTLs específicos para cada uno de los péptidos.

Puesto que estos estudios no pudieron llevarse a cabo utilizando células deficientes en TAP infectadas con vaccinia, no se pudo concluir cuál de las peptidasas identificadas en las células TAP-competentes fue la responsable del procesamiento de los ligandos virales por vías independientes de TAP. Como en estudios previos y en la actual memoria se ha implicado tanto al proteasoma como a las metaloproteasas en la generación de ligandos por vías independientes de TAP cualquiera de las dos vías podría ser la responsable del procesamiento de los epítopos en las células deficientes, aunque también podría existir una tercera vía capaz de generar los ligandos en ausencia de TAP (156).

Un dato relevante sobre los ligandos independientes de TAP es su posible relación con el éxito de la primera vacunación empírica contra la viruela, ya que algunas cepas de cowpox procedentes de la campiña inglesa, región donde comenzó la vacunación, codifican para una proteína CPXV12, capaz de bloquear selectivamente el transporte de péptidos por parte de TAP (21). Por tanto, los epítopos independientes de TAP conservados entre este virus vacunal y viruela pudieron contribuir a la protección cruzada.

A lo largo del siglo XIX las diferentes cepas de cowpox se fueron mezclando con horsepox y vaccinia, de tal manera que incluso en la actualidad la vacuna DRYVAX sigue manteniendo clones con esta proteína que inhibe específicamente la translocación de péptidos. En la actual memoria se han identificado diferentes ligandos independientes de TAP de vaccinia, presentados por alelos de HLA-I predominantes en la población humana, que al estar conservados entre viruela y cowpox podrían haber contribuido a dicha protección cruzada inicial.

Además, cabe destacar que todos los ligandos independientes de TAP que se han podido estudiar en el modelo de ratones transgénicos para HLA (que expresan TAP) son capaces de generar una respuesta de linfocitos T $CD8^+$ en los animales vacunados, por lo que estos epítopos también se estarían generando en células con TAP y por tanto, podrían tener un papel relevante en la respuesta inmune de la población general. La mayoría de estos ligandos virales tienen un tamaño y unos motivos de anclaje similares a los identificados en células que expresan TAP. Sin embargo, la comparación de los repertorios peptídicos independientes de TAP viral con el endógeno presentados en la actual memoria pone de manifiesto que existen algunas características diferentes entre ambos grupos, como son el mayor tamaño y la ausencia de motivos de anclaje en el repertorio endógeno pero no en el viral. Estas diferencias podrían deberse a que los ligandos virales se generen en un compartimento celular distinto al de los endógenos de baja afinidad, en el que exista una mayor competición o al reducido número de ligandos virales identificados. Dado que la replicación de vaccinia tiene lugar en las factorías virales en el citosol y que este virus durante el proceso de morfogénesis utiliza parte de las membranas del retículo y del Golgi, también es posible que algunas proteínas virales accedan a la vía secretoria y sean procesadas allí, proceso que no ocurriría en las células sin infectar.

Finalmente, con respecto a la metodología de los ensayos de inmunoproteómica es de resaltar que la mayoría de los estudios publicados hasta ahora presentan algunas limitaciones, siendo una de las principales la utilización de anticuerpos panespecíficos, reactivos contra todos los alelos de HLA-I presentes en la célula empleada. Ello obliga a la posterior utilización de programas de predicción de epítopos para asignar las distintas secuencias peptídicas obtenidas a cada alelo de HLA de la célula en cuestión (181). El empleo de estos programas tiene ya de entrada una gran limitación, puesto que no permiten la predicción de unión a todos los alelos de HLA y muchas de estas herramientas bioinformáticas únicamente admiten tamaños peptídicos de entre 8 y 10 Aas. Este hecho hace que en los ensayos inmunoproteómicos se descarte como ligandos a péptidos inusuales (largos o sin motivos de anclaje) como el descrito previamente por nuestro grupo (182).

Adicionalmente para comparar esta aproximación metodológica con la desarrollada en la actual memoria, en donde se ha realizado una inmunoprecipitación secuencial de los distintos alelos de HLA, se realizó una asignación bioinformática con los programas SYFPEITHI (http://www.syfpeithi.de), BIMAS (http://www-bimas.cit.nih.gov) e IEDB (http://www.immuneepitope.org) de los 12 ligandos virales identificados por espectrometría de masas en las células deficientes en TAP (Tabla 2). Esta comparación puso de manifiesto que las asignaciones realizadas por los programas de predicción fueron incorrectas para la mitad de los ligandos, bien por asignación errónea, ambigua o incompleta a un determinado HLA. Además, debido a las restricciones de tamaño que presentan, no todos los programas permitieron la evaluación de todos los ligandos. Estas limitaciones de los métodos de predicción y el uso exclusivo de estas herramientas bioinformáticas han podido contribuir también a que en el trabajo de Weinzierl et al. (26) se identificaran un gran número de ligandos de tamaño canónico, procedentes de regiones de secuencia señal, ya que con estos programas informáticos se descartarían ligandos de mayor tamaño, los de baja afinidad de unión (según el programa de predicción) y de moléculas de HLA no analizadas por los algoritmos. Por todo ello, es preferible el empleo de anticuerpos específicos para los distintos alelos de HLA utilizados de manera secuencial, procedimiento realizado en la presente memoria, para no introducir sesgos innecesarios en este tipo de estudios.

Tabla 2.- Comparación de la capacidad de unión de los péptidos virales a los distintos HLA-I predicha por diversas herramientas bioinformáticas y la obtenida experimentalmente.

		HLA	
Ligando	**Secuencia**	**Predicho[a]**	**Experimental**
A17L9-17	MLDDFSAGA	A*02	A*02
A10L614-623	SPEGEETII	B*51	A*02
A10L688-696	ILDRIITNA	A*02	A*02
A10L867-876	SRGYFEHMKK	B*27	B*27
B8R53-59	WQTMYTN	B*27	B*27
ENV57-65	DAKAYDTEV	A*02/C*01	C*01
K2L16-30	YRLQGFTNAGIVAYK	B*27	B*27/C*01
D5R148-157	IAMKRTLLEL	B*51/C*01	B*51
A50R294-301	LPFGSLGI	B*51	B*51
C11R101-110	IPSPGIMLV	B*51	B*51/C*01
A17L9-25	MLDDFSAGAGVLDKDL	A*02/C*01	C*01
D8L112-119	DGLIIISI	B*51	C*01

a: datos obtenidos de las bases de datos SYFPEITHI (http://www.syfpeithi.de), BIMAS (http://www-bimas.cit.nih.gov), and IEDB (http://www.immuneepitope.org)

En conclusión, la identificación de ligandos tanto virales como endógenos para cada uno de los alelos analizados, junto con los resultados obtenidos por otros grupos, pone de manifiesto que las vías de procesamiento independientes de TAP son muy complejas tanto en la cantidad de proteasas implicadas, como en la variedad de sustratos y de ligandos generados en células defectivas en TAP.

VIII.-CONCLUSIONES

1.- Se han identificado 334 ligandos endógenos que presentan mayor longitud y motivos de anclaje más laxos con respecto a los generados en células con TAP. Además, muchos de ellos presentan extensiones en los extremos amino y carboxilo respecto al núcleo peptídico mínimo identificado o se encuentran ubicados en el extremo carboxilo terminal de la proteína parental.

2.- Estos ligandos derivan de 182 proteínas celulares, mayoritariamente citosólicas y nucleares, que podrían estar siendo procesadas en vesículas secretorias.

3.- Las vías de procesamiento independientes de TAP son múltiples y complejas e implican a distintas proteasas en función del alelo analizado: el proteasoma y SP para HLA-A*02 y -B*51 y proteasas lisosomales para HLA-B*27, además de aminopeptidasas inhibibles por leucintiol para todos los alelos estudiados a excepción de HLA-C*01.

4.- Se han identificado 14 ligandos virales generados independientemente de TAP (13 del virus vaccinia y 1 de HIV), presentados por seis moléculas de HLA diferentes.

5.- La mayoría de los ligandos de vaccinia identificados están conservados entre viruela y los distintos agentes vacunales empleados contra este patógeno.

6.- Los 5 ligandos de vaccinia analizados generan respuestas de linfocitos T $CD8^+$ en ratones transgénicos que expresan TAP.

7.- En células TAP-competentes, el proteasoma y/o una o varias metaloendopeptidasas no identificadas intervienen en la generación de los 4 epítopos caracterizados, mientras que distintas aminopeptidasas y una carboxipeptidasa estarían también implicadas en el procesamiento de algunos de estos epítopos virales.

8.- La variedad tanto de ligandos como de epítopos virales identificados junto con su presentación por parte de todos los alelos de HLA analizados permite comprender como los individuos deficientes en TAP, que padecen el Síndrome Linfocitario de Bare tipo I, no presentan una mayor susceptibilidad a padecer enfermedades víricas que los individuos con transportadores TAP funcionales.

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X.-ANEXO I

OTRAS PUBLICACIONES NO INCLUÍDAS EN LA ACTUAL MEMORIA

1. Johnstone, C.*, **Lorente E.***, A. Barriga, E. Barnea, S. Infantes, F. A. Lemonnier, C. S. David, A. Admon, and D. Lopez. 2015. The viral transcription group determines the HLA class I cellular immune response against Human Respiratory Syncytial Virus. *Mol.Cell Proteomics. IN PRESS doi: 10.1074/mcp.M114.045401* ***Equal contribution**

2. Barriga, A., **E. Lorente**, C. Johnstone, C. Mir, V. M. Del, and D. Lopez. 2014. A common minimal motif for the ligands of HLA-B*27 class I molecules. *PLoS One.* 9:e106772.

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